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TESIS DOCTORAL

LIPIDOS BIOACTIVOS EN PRODUCTOS LÁCTEOS: ESTRATEGIAS PARA SU INCREMENTO Y EFECTOS DEL PROCESADO Y LA CONSERVACIÓN.

LUIS MIGUEL RODRÍGUEZ ALCALÁ

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LÍPIDOS BIOACTIVOS EN PRODUCTOS LÁCTEOS: ESTRATEGIAS PARA SU INCREMENTO Y EFECTOS DEL PROCESADO Y LA CONSERVACIÓN.

Memoria presentada por

LUIS MIGUEL RODRÍGUEZ ALCALÁ

Para optar al grado de

Doctor en Ciencia y Tecnología de los Alimentos

Dirección: Dr. Fco. Javier Fontecha Alonso.

Instituto del Frío (CSIC)

FRANCISCO JAVIER FONTECHA ALONSO, DOCTOR EN CIENCIAS QUÍMICAS E INVESTIGADOR CIENTÍFICO DEL INSTUTO DEL FRÍO DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

CERTIFICA:

Que el trabajo titulado: “LIPIDOS BIOACTIVOS EN PRODUCTOS LACTEOS: ESTRATEGIAS PARA SU INCREMENTO Y EFECTOS DEL PROCESADO Y LA CONSERVACION”, que constituye la Memoria de la que es autor Luis Miguel Rodríguez Alcalá, ha sido realizada en el Instituto del Frío del C.S.I.C. bajo mi dirección y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Autónoma de Madrid y, por tanto, autorizo su presentación.

Y para que conste a los efectos oportunos, firmo el presente Certificado en Madrid a 16 de Noviembre de 2009

Francisco Javier Fontecha Alonso

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A mi familia,

todo esto es gracias a vosotros.

Los grandes saltos se consiguen a través de pequeños brincos.

LISTA DE ABREVIATURAS

AA: ácido araquidónico

Ag^{+}-HPLC: cromatografía de líquidos con columnas de plata ligada.

CLA: ácido linoleico conjugado

CLnA: ácido linolénico conjugado

DHA: ácido docosahexanoico

ELSD: detector “evaporative light scattering”

EPA: ácidos eicosapentanoico

FFA: ácidos grasos libres

FID: detector de ionización de llama

GC: cromatografía de gases

LA: ácido linoleico

LNA: ácido linolénico

LPC: lisofosfatidilserina

MFGM: membrana del glóbulo graso de la leche

MG: monoglicérido

MUFA: ácidos grasos monoinsaturados

PA: ácido fosfatídico

PC: fosfatidilcolina

PE: fosfatidiletanolamina

PI: fosfatidilserina

PLs: fosfolípidos

PS: fosfatidilserina

PUFA: ácidos grasos poliinsaturados

RA: ácido ruménico

SFA: ácidos grasos saturados

SP: esfingomielinas

TFA: ácidos grasos *trans*

TG: triglicérido

TVA: ácido trans vacénico

ÍNDICE

INTRODUCCIÓN

1. INTRODUCCIÓN

La idea según la cual, la salud puede ser mejorada o preservada a través de la alimentación no es un término ideado en el siglo XX. Ya desde la antigüedad se tenía constancia de ello, como así lo atestiguan las palabras de Hipócrates (siglo.V. aC): "*Que tu alimento sea tu medicina y que tu medicina sea tu alimento*".

No obstante, es a partir de la segunda mitad del siglo XX, cuando el concepto se materializa en productos y/o suplementos alimenticios cuya intención clara es provocar un efecto positivo sobre el estado de salud del consumidor y que han supuesto un descenso en la morbilidad y mortalidad, así como un incremento en la calidad de vida de la población (Jones & Jew, 2007).

Fue en Japón en 1980, con el progresivo envejecimiento de la población y buscando disminuir el gasto sanitario en geriatría, cuando el Ministerio de salud nipón inicia y promueve estudios para el desarrollo de estos alimentos "funcionales" (Arai, 1996).

El concepto incluía la relación y equilibrio entre el cumplimiento de los requerimientos en nutrición, la satisfacción sensorial y la modulación de los sistemas fisiológicos del consumidor, entendiendo estos alimentos como un remedio o forma de prevención y no un fármaco para el tratamiento de la enfermedad, como lo muestran las legislaciones aprobadas para crear una nueva categoría de alimentos (FOSHU; food for specific health uses) y el establecimiento de las alegaciones permitidas, en los productos japoneses (Menrad, 2003).

En la Unión europea, se consideran alimentos funcionales aquellos que además de nutrir, afectan satisfactoriamente a una o más funciones corporales o fisiológicas, además de prevenir la incidencia de enfermedades crónicas (Urala & Lahteenmaki, 2007).

La importancia de este tipo de alimentos queda claramente puesta de manifiesto, a tenor de su impacto económico, que en el mercado mundial de los funcionales fue en 2007 de 56 millones de € (21 en EE.UU, 7 en Europa, 28 en

Asia) y se espera que para 2012 sea de aproximadamente de 74 millones de € (Datamonitor analysis, 2007)

1.1 LÍPIDOS LÁCTEOS Y SALUD

Las recomendaciones nutricionales relativas a la dieta de los países industrializados, inciden en la necesidad de reducir el consumo total de grasa, principalmente saturada, (SFA) ácidos grasos *trans* (TFA) y de colesterol (Jenkins & McGuire, 2006; Kris-Etherton & Innis, 2007) ante el hecho del incremento en enfermedades cardiovasculares y su relación con la dieta actual. La Organización mundial de la salud recomienda que el consumo de grasas no debe representar más del 15-30% del aporte energético, no excediendo el 10% para SFA, 6-10% de poliinsaturados (PUFA) (8-5% de ω6 y 2-1% de ω3), <1% de *TFA*, mientras que el aporte de monoinsaturados (MUFA) se indica como el resultante de la diferencia entre grasa total y la suma de SFA, PUFA y *trans* (WHO, 2008).

Sin embargo, no debe olvidarse que los lípidos como macronutrientes que son, presentan además de las funciones de suministro energético y protección de órganos, y otras como ser fuente de ácidos grasos esenciales, vehículo de vitaminas liposolubles A, D, E y K, facilitando su absorción, y de otros compuestos antioxidantes.
Los SFA, son característicos de alimentos de origen animal (Bessa *et al.*, 2007), mientras que los *trans*, se encuentran en mayor concentración en aceites vegetales y de pescado, hidrogenados industrialmente (Pfeuffer & Schrezenmeir, 2006). En ambos casos está demostrado que su consumo excesivo se relaciona con el desarrollo, en humanos, de enfermedades cardiovasculares (CVD), obesidad, síndrome metabólico e incluso ciertos tipos de cáncer (Warensjo *et al.*, 2006; Jacobson *et al.*, 2007; Shannon *et al.*, 2007).

La grasa láctea, dado su origen animal contiene altas concentraciones en SFA (Tabla 1) y presencia de colesterol, por lo que pese al gran valor nutricional de la leche, es percibida por parte de los nutricionistas y consumidores, como un

alimento no del todo saludable. No obstante, en el momento actual, el conocimiento adquirido a través de diversas investigaciones conduce a pensar que determinados ácidos grasos y componentes, presentes en la grasa láctea, juegan un rol muy beneficioso en la salud (Parodi, 2004), como es el caso de los ácidos grasos butírico (C4), *trans* vacénico (C18:1 t11, TVA) y ácido linoleico conjugado (CLA) con actividades anticancerígenas (Blank-Porat *et al.*, 2007; Soel *et al.*, 2007); oleico (C18:1 c9; OA) y linoleico (C18:2 c9,c12; LA) antiaterogénicas (Pérez-Jiménez *et al.*, 2007) y los omega 3, muy importantes en el desarrollo del sistema nervioso infantil (Fleith & Clandinin, 2005) y mejora de ciertos trastornos mentales (Appleton *et al.*, 2006), además del efecto como protector cardiovascular (Calder, 2006).

Tabla 1. Cotenido medio (% p/p) en ácidos grasos mayoritarios en grasa láctea de leche de vaca, cabra y oveja.

Átomos de carbono	Ac. Graso	Vaca	Oveja	Cabra
C4: 0	Butírico	2–5	3,1–3,9	2–2,4
C6: 0	Caproico	1–5	2,7–3,4	2–2,7
C8: 0	Caprílico	1–3	2,1–3,3	2,3–3
C10: 0	Cáprico	2–4	5,5–9,7	8,8–11
C12:0	Láurico	2–5	3,5–4,9	3,9–6,2
C14: 0	Mirístico	8–14	9,8–10,7	7,7–11
C15: 0	Valérico	1–2	0,9–1,1	0,5–0,8
C16: 0	Palmítico	22–35	22,5–28,2	23–34,8
C16:1	Palmitoleico	1–3	0,7–1,3	1–2,7
C17: 0	Margárico	0.5–1.5	0,6–0,7	0,5–0,9
C18: 0	Esteárico	9–14	8,5–11	5,8–13,2
C18:1	Oleico	20–30	17,8–23	15,4–27,7
C18:2	Linoleico	1–3	2,9–3,7	2,5–4,3
C18:3	Linolénico	0.5–2	0,5–1	0,2–0,9
18:2 conj	CLA	0,3-1	0,6–1	0,3–1,2

Datos: Jahreis *et al.*, 1999; Ritzenhaler *et al.*, 2001; Jensen, 2002, Park et al. 2007.

Incluso en el grupo de SFA no todos tienen relación con las enfermedades cardiovasculares: butírico (C4), caproico (C6), caprílico (C8), cáprico (C10) y esteárico (C18), resultan ser positivos o neutros, mientras que láurico (C12), mirístico (C14) y palmítico (C16) parecen en cierta medida responsables del

incremento en el riesgo cardiovascular (Thijssen *et al.*, 2005; Mensink, 2006). No obstante ciertos estudios revelan que aunque C12, C14 y C16 muestren propiedades aterogénicas y aumenten los niveles del Colesterol LDL, también contribuyen a aumentar las concentraciones del Colesterol HDL (Steijns, 2008; Parodi, 2009), mientras que en otros, la presencia de insaturados reducirían los efectos del C16 (Clandinin *et al.*, 2000) y en general no dejan clara la relación de las enfermedades cardiovasculares y los ácidos grasos saturados.

Una explicación a las discrepancias encontradas entre los diferentes estudios, frente a los marcadores de CVD, es debida al uso de fórmulas que incorporan grasas sintéticas, con ácidos grasos esterificados al azar. En contraste, en la grasa láctea, la distribución de los ácidos grasos en los triglicéridos (TG) es relativamente constante y característica. La mayoría de los SFA se localizan en la posición *sn*-2 del TG (principalmente el ácido palmítico), los ácidos grasos de cadena corta (SCFA) se encuentran mayoritariamente en posición *sn*-3, el esteárico y oleico en *sn*-1 y *sn*-3 (Jensen, 2002). La acción de las lipasas lingual, gástrica y pancreática, estereoespecíficas de los enlaces *sn*-1 y *sn*-3, contribuye a la liberación de SCFA, así como los monoglicéridos derivados *sn*-2, que junto con el calcio de la leche permite que su absorción se produzca de una manera más eficaz (Mensink, 2005). Por otra parte, la exclusiva presencia en grasa láctea de SFA de cadena corta, butírico, caproico y de cadena media, caprílico y cáprico, que constituyen del 8 al 12 % del total, no tienen efecto sobre los niveles del colesterol en sangre (Parodi, 2004). Además, la presencia de estos SCFA, supone un elevado contenido en TG de cadena corta y media, lo que favorece su punto de fusión más bajo. Estas diferentes propiedades químicas y físicas, frente a otras grasas animales saturadas, afectan de manera positiva a su digestibilidad y favorece su biodisponibilidad. El ácido esteárico con un contenido medio del 12%, es considerado neutro desde la perspectiva de la salud humana, aunque sin duda es tan efectivo para reducir el colesterol plasmático como el ácido oleico, también presente en grasa láctea en concentraciones altas, del 15 al 23%. Es de interés, asimismo, considerar la importante presencia de linoleico, y α-linolénico, con un 3% y 0.7% respectivamente, de reconocido efecto positivo para la salud cardiovascular. La grasa de leche tiene además ácidos metíl-ramificados, sobre todo la leche de

cabra, cuya relevancia se debe fundamentalmente a sus propiedades anticancerígenas descritas en cultivos de células tumorales, su influencia en el punto de fusión de la grasa láctea y por su utilidad en estudios clínicos como marcadores del consumo humano de grasa láctea, al no encontrarse en otras grasas animales (Vlaeminck *et al.*, 2006)

El motivo de la asociación entre consumo de productos lácteos y CVD es debido a que la grasa saturada incrementa en humanos los niveles de colesterol (Mensink *et al.*, 2003a). Sin embargo, diversos estudios ponen en duda esta idea. En datos procedentes de 12 ensayos de cohortes (>280.000 sujetos) y tiempos de estudio de 20 años, en 5 de ellos no encontró una asociación directa entre la ingesta de productos lácteos y CVD (German *et al.*, 2009). En otros ensayos clínicos llevados a cabo con 28.886 mujeres con edades por encima de los 45 años y comparando los posibles efectos de una dieta que incluía lácteos de bajo contenido graso frente a otra de alto contenido, encontraron que el consumo de los lácteos de bajo contenido graso estuvo asociado a una disminución del 11% en el riesgo de padecer hipertensión (Wang *et al.*, 2008), mientras que con lácteos de alto contenido graso no pudo encontrarse ninguna asociación.

El síndrome metabólico (MS) ha emergido recientemente como un concepto clínico con importantes implicaciones en términos de riesgo de desarrollo de CVD. La prevalencia del MS ha sido estimada en torno al 25% en países industrializados (Ford *et al.*, 2002). En muchos estudios el consumo de lácteos se ha asociado inversamente con la aparición de uno o más facetas del MS. En el estudio CARDIA, que fue llevado a cabo con 3157 adultos de edades comprendidas entre los 18-30 años y comparando un consumo de productos lácteos $\geq$ 5 raciones/día frente a <1.5 raciones/día, se observó una reducción del 70% en el riesgo de desarrollo del MS en un periodo de 10 años (Pereira *et al.*, 2002).

Los ácidos grasos *trans* producidos industrialmente a partir de aceites vegetales y de pescado parcialmente hidrogenados, han demostrado en

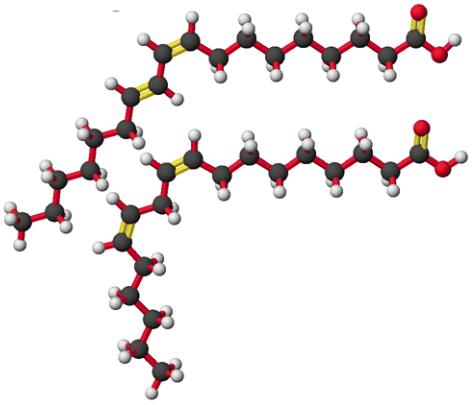
conjunto aumentar el riesgo de enfermedades coronarias, ya que tienen influencia adversa sobre la relación de LDL/HDL (Ascherio *et al.*, 1999; Lichtenstein *et al.*, 2003; Mensink *et al.*, 2003b). El perfil de estos isómeros de ácidos *trans* tiene una distribución gausiana con niveles altos de los isómeros *trans*-9, *trans*-10 y *trans*-11, muy diferente del perfil presente en la grasa de leche. No obstante, algunos trabajos han cuestionado si el TVA tiene esos mismos efectos adversos. Así, Meijer *et al*. (2001) encontró que TVA fue más perjudicial para el riesgo cardiovascular que el ácido elaídico (C18:1 t9) debido a que provocaba un mayor aumento de la relación LDL/HDL. Además, Clifton *et al*. (2004) demostraron que el TVA actúa como marcador independiente del infarto de miocardio. En contraste con estos resultados, se ha demostrado más recientemente que los ácidos *trans* de origen animal no dan lugar a un mayor riesgo de cardiopatía coronaria (Willett, 2006; Willett & Mozaffarian, 2008) y que además el consumo medio en una dieta europea es bajo, con una contribución de alrededor del 0.7% del total energético. En esta misma línea, Tricon *et al.* (2006), demostraron que un aumento de la concentración de TVA y C18:2 c9, t11 CLA en la grasa láctea no se relaciona con efectos perjudiciales en la mayoría de las enfermedades cardiovasculares. Cardigny *et al.*(2008) en estudios con 46 individuos (22 hombres y 24 mujeres) donde se comparó el consumo de TFAs de origen industrial frente a las procedente de fuentes naturales (11-12 g/d) encontraron que en mujeres los niveles de HDL y apoA1 aumentaron significativamente por efecto de los TFA de origen animal, aunque también se encontró un incremento en las concentraciones de las partículas LDL de mayor tamaño y más ligeras así como de su apolipoproteinas apoB. Estos resultados también fueron observados en otros trabajos (Motard-Belanger *et al.*, 2008).

Sin embargo, el consumo de TFAs ruminales no alcanzan nunca los niveles empleados en los trabajos expuestos y que implicarían un consumo diario de 10 raciones de queso, 5 de leche entera, 2 de yogur entero y 20 cucharas de mantequilla (German *et al.*, 2009).

Por todo ello, sigue siendo necesario la realización de más estudios para aclarar los efectos que sobre los lípidos sanguíneos producen los distintos isómeros *trans* C18:1 (IDF, 2005).

Pero sin duda es el ácido ruménico (C18:2 c9,t11, RA), isómero mayoritario del CLA, el que más atención, debates e investigaciones está generando de entre los ácidos grasos presentes en grasa láctea,. Los distintos isómeros de CLA se caracterizan por la presencia en su cadena de 18 átomos de carbono, de dos dobles enlaces conjugados y por estar presentes principalmente en carnes de rumiantes y especialmente en la leche de estos animales (Fritsche *et al.*, 1999; Schmid *et al.*, 2006).

Figura 1. Estructura del ácido linoleico (LA) y del ácido linoleico conjugado (CLA)



Se ha descrito que los dobles enlaces pueden encontrarse entre las posiciones 6-14 (6,8; 7,9; 8,10; 9,11; 10,12; 11,13; 12,14), con 4 isomerías geométricas (*trans,trans, cit,trans-trans,cis y cis,cis)*; dando un total de 28 isómeros posibles (Roach et al., 2002). De ellos, el más abundante es el RA, el cual representa el 75-90% del total de CLA en grasa láctea (Collomb et al., 2006), seguido del isómero C18:2 t7,c9 que puede encontrarse en un rango de 3-16% (Chin et al., 1992; Tanaka, 2005).

Estos ácidos grasos fueron descritos en grasa láctea por primera vez por Booth *et al.* (1935), despertándose el interés generalizado en ellos tras los estudios de Ha *et al.* (1987) donde se describía su capacidad anticancerígena por la inhibición de tumores epiteliales en animales de experimentación. Desde entonces, numerosos estudios han profundizado en sus efectos biológicos (Tablas 2 y 3), informándose de capacidades antiaterogénicas (Tanaka, 2005), antioxidantes (Hur *et al.*, 2007), mejora del sistema inmunológico (O'Shea *et al.*,

2004) y de reducción de la grasa corporal (Park & Pariza, 2007) por el isómero C18:2 t10,c12, presente en grasa láctea en concentraciones inferiores al 1%, pero en elevadas concentraciones en aceites ricos en CLA por síntesis química (ej. Tonalin®).

Tabla 2. Efectos biológicos generales del CLA.

Sujeto	Efecto	Referencia
Hamsters	Mejora en ECV: expresión de genes pro-inflamación.	Toomey et al., 2006
Cultivos celulares	Apoptosis (Adipocitos y células cancerosas)	Miglietta et al., 2006 Tsuboya et al. 2000
Ratas	Inhibicion tumoral (proliferación tumoral)	Kim et al., 2005 Ohysu et al., 2005
Ratones	Mejora de la insulinodependencia	Zhou et al, 2008
Ratas	Mejora de la respuesta inmune	Ramirez-Santana et al. 2009,
Ratones	Reducción de la grasa corporal	Parra et al., 2009

Actualmente existen dos teorías propuestas para explicar los efectos biológicos del CLA (Wall *et al.*, 2008). En la primera se sostiene que los isómeros del ácido linoleico conjugado CLA disminuyen las concentraciones del ácido araquidónico en los fosfolípidos de las membranas celulares, reduciendo la acción de compuestos derivados de los eicosanoides tales como prostaglandinas y leucotrienos. La explicación alternativa, sitúa al CLA como regulador de genes implicados en el metabolismo lipídico, apoptosis, funciones del sistema inmune y balance energético. Se ha sugerido que el CLA compite con el ácido araquidónico (C20:4) en la reacción de la ciclooxigenasa, lo que reduce la concentración de prostaglandinas y tromboxanos de la serie 2 además de poder suprimir la expresión de genes de la ciclooxigenasa y reducir la liberación de citoquinas pro-inflamatorias tales como TNF-alfa e interleukina en animales (Akahoshi *et al.*, 2004). El CLA también parece activar los factores de transcripción PPARs, reducir el paso inicial en la activación del NF-kappa B y por tanto reducir las citoquinas, moléculas de adhesión, y de otros tipos de moléculas inducidas por estrés (Cheng *et al.*, 2003).

Tabla 3. Estudios relativos a los efectos biológicos del CLA en humanos.

	Dosis	Sujeto	BMI medio	Suplemento	Duración	Efecto	Referencia
Efecto sobre la composición corporal	1,7-6,8 g/d	Humanos	25-35	Tonalin®(75% CLA)	12 semanas	Si	**Blankson et al,2000**
	0,6 g/d	Humanos	25	Tonalin®(60% CLA)	12 semanas	Si	**Thom et al,2001**
	3,6-3,4 g/d	Humanos	25-30	FFA-TG (Natural lipids)	12 meses	Si	**Gaulier et al,2004**
	2,2-4,2 g/d	Humanos	25	Capsulas	4-12 semanas	Si	**Risérus et al, 2004**
	3,0-1,5 g/d RA ó 10t,12c	Humanos	25-30	Bebible lácteo (Natural lipids)	18 semanas	No	**Malpuech-Brugére et al,2004**
	1,5 (CLA)-1,42 RA g/d.	Humanos	25	P. lácteos naturales	6 semanas	No	**Tricon et al, 2006**
Inhibición de crecimiento tumoral	0-4mm/L	Células tumorales (Colón)		Isómeros		SI	**Han et al, 2006**
	100 mM	Células tumorales (Humanas)		Extractos lipídicos de carne		SI	**De la Torre et al,2006**
Cáncer rectal	Ingesta diaria	Mujeres		Leche y carne	3 + 7 años	SI	**Larsson et al, 2005**
Cáncer de mama	Ingesta diaria	Mujeres		Leche y carne	6 años	SI	**Voorrips et, 2002**

Otros componentes de la fracción lipídica de la leche, se han descrito también por sus importantes actividades biológicas. La membrana del glóbulo graso de la leche (MFGM, Figura 2.) está compuesta por lípidos y proteínas procedentes de las células epiteliales de la glándula mamaria, e incluyen cantidades significativas de fosfolípidos (PLs). Aunque los PLs constituyen un porcentaje pequeño de los lípidos totales, 0.3-1% en leche y derivados (Tabla 4) y 0,3% en leche humana, se encuentran altamente implicados en el metabolismo celular debido a su carácter lipofílico e hidrofílico. Entre los PLs presentes en el glóbulo graso, destacan la fosfatidilcolina (PC) 35%, fosfatidiletanolamina (PE) 30%, esfingomielina (SM) 25%, fosphatidilinositol (PI) 5% y fosfatidilserina (PS) 3% (Sánchez-Juanes *et al.*, 2009).

Entre las actividades biológicas descritas para los PLs destacan su carácter antioxidante (Frede *et al.*, 1990; Saito & Ishihara, 1997), propiedades antimicrobianas y antivirales (Van Hooijdonk *et al.*, 2000), así como efectos protectores frente a la úlcera gástrica (Kivinen *et al.*, 1992). Estudios recientes han demostrado que los PLs parecen desarrollar importantes funciones como agentes activos frente al cáncer de colon, frente a patógenos gastrointestinales, Alzheimer, depresión y estrés, además tener un efecto nutricional positivo en la reducción del riesgo de CVD (Spitsberg, 2005), permitiendo considerar la MFGM como un potencial nutracéutico o ingrediente nutricional.

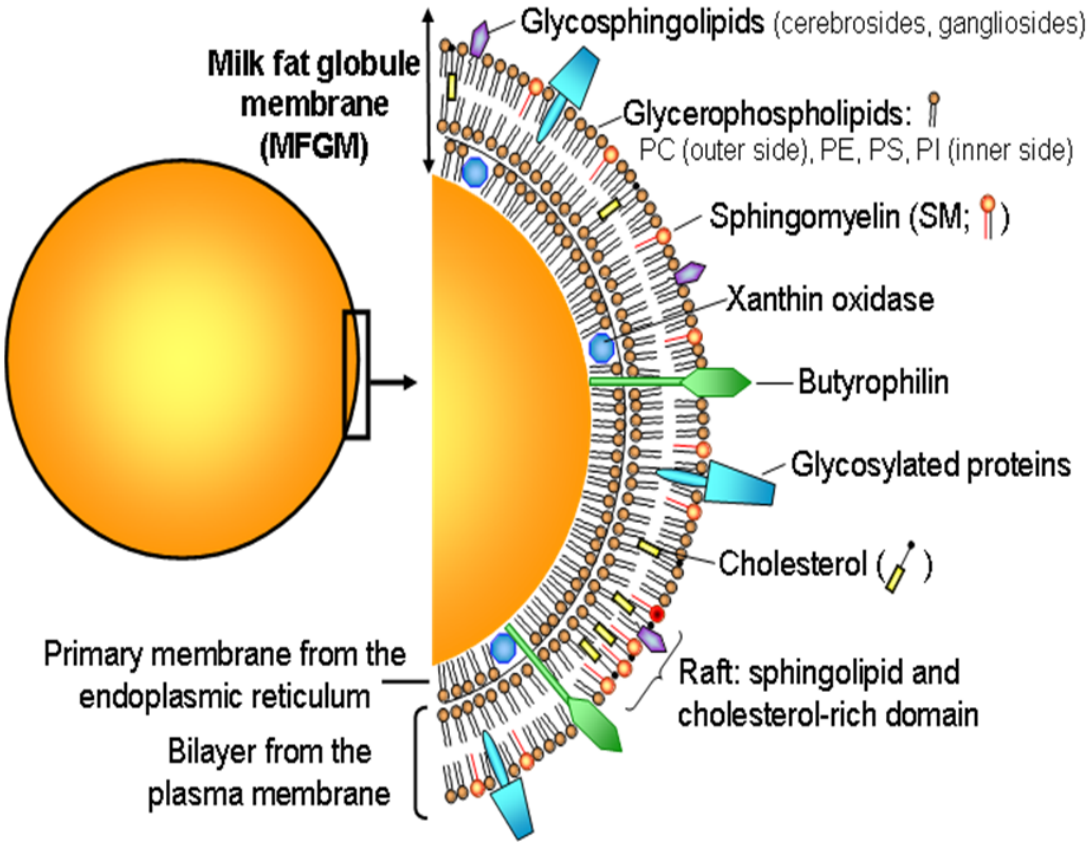
Por lo tanto, nos encontramos ante un alimento, leche y productos lácteos, con una calidad nutricional notable, del que su fracción lipídica cuenta con componentes que sería de interés disminuir su contenido y otros en los que el beneficio vendría del aumento de sus concentraciones.

Tabla 4. Contenidos y distribución de lípidos polares en productos lácteos y derivados.

Producto	Extrato seco (ES)	Grasa	Lipidos Polares (contenidos)			Lipidos polares (composición)						
	g/100g	g/100g	mg/100g	g/100g ES	g/100g grasa	PE	PS	PI	PC	GluCer	Laccer	SM
Leche Cruda	12,6	4,15	29,4	0,23	0,7	42	6,7	4,8	19,1	2,7	6,7	17,9
Leche Pasteurizada semidesnatada.	10,6	1,46	18,8	0,18	1,3	35	8,9	7,9	20,2	4,8	6,2	17
UHT entera	12,48	3,47	21,2	0,17	0,6	34	9,1	7,9	20,5	2,9	6,2	19,5
UHT semidesnatado	10,52	1,53	14,2	0,13	0,9	33	7,9	4,8	22	2,2	7,2	22,9
UHT desnatada	9,18	0,12	12,8	0,14	10,7	38,2	9,9	5,5	19,6	1,8	8,3	16,7
Esterilizada semidesnatado	10,18	1,57	16	0,16	1	34,3	7,7	5,1	24,2	3	8,3	17,4
Nata pasteurizada	45,15	39,23	138,9	0,31	0,4	40	8,2	8,2	20,7	3,7	5,3	13,9
Leche condensada	26,67	7,8	75,1	0,28	1	34,6	8,6	8,5	22,2	1,5	7,6	16,9
Mantequilla	84,81	83,09	141	0,17	0,2	37,6	8,2	4,3	20,9	3,6	8,1	17,2
Mazada dulce	8,01	0,42	91,8	1,15	21,8	42,9	8,6	8,9	19,1	1,6	6,1	12,8
Mazada fermentada	7,69	0,5	115,5	1,5	23,1	44,8	9,1	6,8	17,3	2,2	7,6	12,1
Yogur desnatado	9,27	0,32	17,9	0,19	5,5	31,1	7,9	6,3	19,9	2,6	7,4	24,9
Kefir semidesnatado	10,16	1,5	34	0,33	2,3	40,3	7	5,5	20,7	2	6,3	18,3
Queso Ricotta	24,08	10,36	278,8	1,16	2,7	45,4	5,8	4,4	15,8	3	11,3	14,2
Queso Quark desnatado	13,16	0,13	32,4	0,25	24,7	39,1	5,9	3,9	18,7	2,6	10,4	19,4
Queso Quark	19,35	6,83	58,1	0,3	0,9	40,1	7	5,6	19,1	3,2	6,3	18,8
Queso Cottage	20,18	4,4	55,8	0,28	1,3	34,2	9,5	9,4	21,7	2	6,6	16,7
Queso fresco	37,58	27,05	148,9	0,4	0,6	39,9	8,2	6,5	21,7	3,4	6,1	14,1
Queso Mozzarella	40,48	23,54	115,3	0,28	0,5	42,5	5,6	5,7	19,4	4,5	7,8	14,6
Queso Gouda desnatado (8 semanas)	54,22	21,1	93,9	0,17	0,4	39,1	7,7	6,1	20,5	1,9	6,1	18,5
Queso Gouda (8 semanas)	58,41	30,8	151	0,26	0,5	39,2	7,2	6,8	20,6	1,8	9	15,5
Queso Gouda (36 meses)	70,51	36,13	147,1	0,21	0,4	36,8	8,6	7,9	19,9	1,3	7,6	17,9
Queso Cheddar	62,05	32,51	153,9	0,25	0,5	38	8,5	7,7	20,3	2,4	6,9	16,3
Queso Camembert	50,56	24,13	123,3	0,24	0,5	28,3	8,6	6,7	22,8	5,9	12,7	14,9
Queso Emmenthal	61,88	26,68	109,7	0,18	0,4	36,9	8,9	6,5	21,2	3,8	9,1	13,5
Queso Parmigiano (24 meses)	71,43	30,41	110,9	0,16	0,4	24,6	7,9	5,3	19,7	5,1	11,7	25,7
Queso Mozzarella (suero)	6,7	0,31	19,1	0,29	6,2	40,6	9,3	4,6	19,1	2,3	8,5	15,7
Queso Cheddar (suero)	6,65	0,33	17,6	0,26	5,3	41,1	9,3	3,7	19	1,6	8,9	16,4

Datos: Rombaut, 2007. PE: fostidilestanolamina, PS: fosfatidilserina,PI: fosfatidilinositol, PC: fosfatidilcolina, Glucer: glucocerebrósido, Laccer: lactocerebrosido, SM: esfingomielina.

Figura 2. Representación esquemática de la estructura de la membrana del glóbulo graso (con permiso de la Dra. Christelle Lopez, INRA-Agrocampus, Rennes, Francia).



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1.2 MEJORA DE LA COMPOSICION DE LA GRASA LACTEA.

Con el objetivo de mejorar la calidad de nutricional de la grasa láctea, de entre los diferentes métodos industriales (eliminación, sustitución o fortificación) y zootécnicos, estos últimos simplifican el proceso de elaboración del producto y actualmente es posible que los rumiantes produzcan leche con grasa más insaturada, en base a la composición lipídica de las dietas que reciben y al conocimiento de los procesos metabólicos por los que el animal es capaz de incluirlos en las fracciones lipídicas de la carne y la leche. Estos procesos se basan en la biohidrogenación de los PUFA que tienen lugar en el rumen y de la desaturación de TVA y otros ácidos grasos en glándula mamaria.

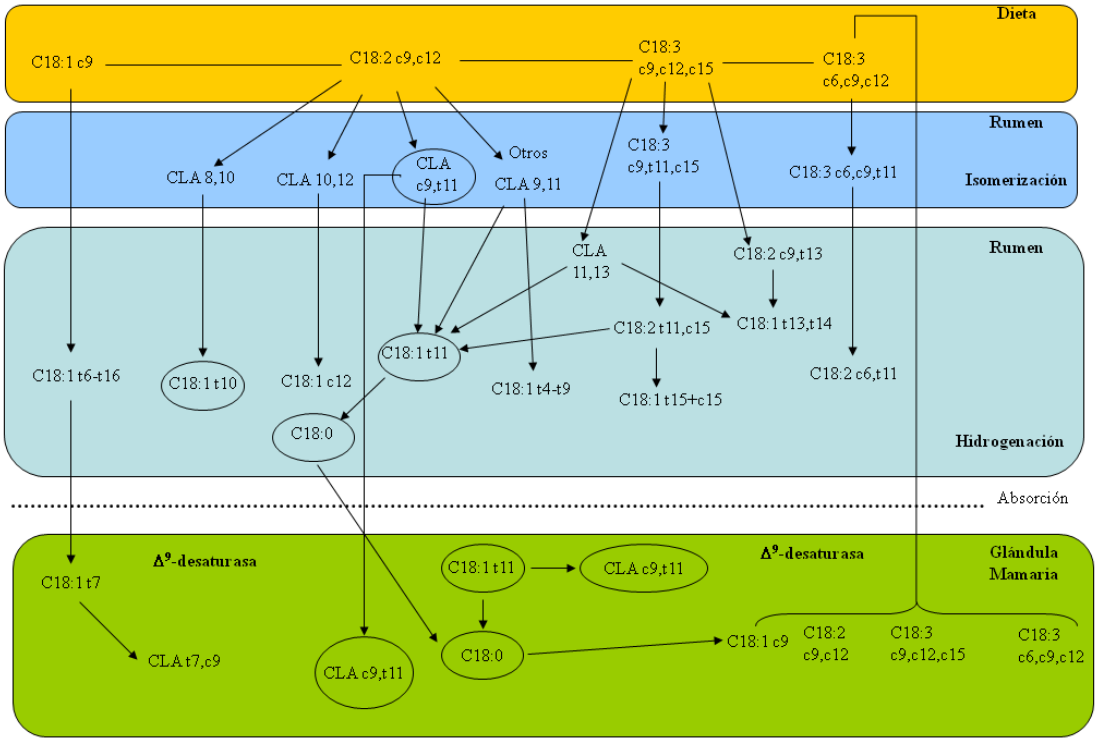
1.2.1 Biohidrogenación en el rumen de ácidos grasos poliinsaturados

La biohidrogenación de los PUFA Es un proceso anaerobio llevado a cabo por enzimas bacterianas de *Butyrivibrio fibrisolvens*, que tiene lugar en el rumen del animal. Como sustrato son empleados mayoritariamente linoleico y linolénico (Figura 2), procedentes de la dieta (Bauman & Griinari, 2003; Sieber *et al.*, 2004; Jouany *et al.*, 2007). En una primera etapa, las enzimas producidas, isomerizan los dobles enlaces *cis* 12 en *trans* 11 y 13, para, tras una serie de hidrogenaciones y reducciones, llegar a la aparición de isómeros de C18:1 (Destaillats *et al.*, 2005b; Bessa *et al.*, 2007).

Estas reacciones dan lugar a la producción de una gran variedad de ácidos grasos, generalizándose que los principales compuestos obtenidos son TVA y RA (Chilliard *et al.*, 2007). En un principio se pensó que este isómero de CLA procedía exclusivamente de la biohidrogenación en el rumen, de ahí su nombre (Kramer *et al.*, 1998); sin embargo, estudios llevados a cabo mediante infusión abomasal en vacas demostraron que era la glándula mamaría el lugar donde tiene lugar alrededor del 75% de su producción (Griinari *et al.*, 2000). Llegados a este punto, los ácidos grasos pueden ser nuevamente reducidos por desaturasas de un segundo grupo de bacterias hasta convertirlo en esteárico (Bauman *et al.*, 2006).

Fuera del rumen, los ácidos grasos, llegarán al torrente sanguíneo, pudiendo ser absorbidos por la glándula mamaria, donde se iniciará una nueva serie de reacciones y finalmente incorporados a la leche. Los ácidos oleico, TVA, esteárico, C18:1 t13 y C18:1 t7, son reducidos por la enzima Δ^9-desaturasa, dando lugar a RA y C18:2 t7,c9 entre otros. Esta enzima puede igualmente actuar sobre mirístico, palmítico y otros monoinsaturados como C18:1 t12 (Destaillats *et al.*, 2005b). Corl *et al.* (2002) demostraron que el segundo isómero más abundante de CLA, el C18:2 t7,c9 , también debe su presencia a la inclusión de dobles enlaces por enzimas de la glándula mamaria. Además de las reacciones descritas, también tiene lugar la síntesis *de novo* de ácidos grasos, para dar lugar a la formación de compuestos de cadena corta y media, cuyas rutas pueden ser inhibidas por los compuestos producidos durante la biohidrogenación (Chilliard *et al.*, 2007; Moate *et al.*, 2007).

Figura 3. Rutas de biohidrogenación en el rumen y en glándula mamaria.



(Corl *et al.*, 2002; Destaillats *et al.*, 2005b; Dhiman *et al.*, 2005; Collomb *et al.*, 2006; Jenkins & McGuire, 2006; Chilliard *et al.*, 2007; Wallace *et al.*, 2007).

Cuando no es posible ofrecer al rumiante dietas basadas en pastos frescos, para asegurar el aporte de ácido linoleico (C18:2 c9,c12) y linolénico (C18:3 c9,c12,c13), se recurre generalmente a la suplementación de la dieta con semillas y/o aceites vegetales. Sin embargo, la cubierta de las semillas puede dificultar la biohidrogenación en el rumen, al evitar que las enzimas bacterianas entren en contacto con los lípidos, por lo que se recurre a la extrusión de las semillas o a la adición de aceites como fuentes de ácidos grasos poliinsaturados.

Como se ha indicado anteriormente, el ácido linoleico es el precursor principal para la síntesis de RA y TVA en grasa láctea; sin embargo, el linolénico también promueve la presencia de otros compuestos poliinsaturados de interés biológico por la presencia de dobles enlaces conjugados y tratarse de ácidos omega 3, los isómeros del linolénico conjugado, también conocidos por el acrónimo CLnA (Tsuzuki *et al.*, 2004),. Suplementar las dietas es una práctica común en ganadería ya que permite incrementar el aporte energético al animal, mejorando su capacidad productiva (Schroeder *et al.*, 2003). Además, el empleo de fuentes lipídicas ricas en PUFAs permite obtener productos de origen animal cuya grasa es más insaturada (Schroeder *et al.*, 2004).Diversos trabajos y revisiones (Demeyer & Doreau, 1999; Baer *et al.*, 2001), mostraron que el contenido en saturados puede representar el 60-70% del total de la composición de la grasa láctea de leche de vaca, cuando la alimentación se basa en ensilados de cereales, mientras que la suplementación con semillas de alto contenido en linoleico y α-linolénico, los valores pueden ser del 50% en SFAs (en función del tratamiento previo de las semillas para hacer más fácil su digestión). Empleando pastos los contenidos en SFA pueden llegar al 57%, aunque otros autores (Loor *et al.*, 2002) han reportado menores concentraciones (8-28%). Por otra parte, el empleo de suplementación con aceites de pescado produce una disminución del contenido de saturados hasta el 40-50% y un aumento apreciable del contenido en ácidos grasos eicosapentanoico (EPA) y docosahexanoico (DHA). En estos mismo trabajos se ha descrito que el contenido total en ácidos grasos monoinsaturados y poliinsaturados puede variar entre el 20-27% (Oleico, 16-24%, linoleico 1.7-2%) en dietas basadas en ensilados de cereales, 35-45% (12-35% oleico; 2-4% linoleico) en dietas suplementadas con semillas oleaginosas y 23% (6% de oleico, 1.7% de linoleico) para aceites de pescado, mientras que fue del

42% de (28.5% de oleico, 1.8% de linoleico) cuando la alimentación se basó en pastos.

Tal como se ha indicado anteriormente, el rumen contribuye de forma limitada al contenido final del CLA en la leche. Es por ello que la actividad enzimática endógena ha recibido gran atención dentro del desarrollo de estrategias para aumentar el contenido en CLA en grasa láctea. Diversos estudios concluyen que los mejores resultados se obtienen cuando se suplementa con fuentes lipídicas (aceites y semillas de lino, girasol y soja) y pastos, mientras que la utilización de aceites de pescado y constituyentes grasos protegidos, como las sales cálcicas, producirían alteraciones en el rumen, dando lugar a una biohidrogenación incompleta de los ácidos grasos insaturados y en ciertos casos disminución del contenido en ácidos grasos de cadena corta y media (de la Fuente & Juarez, 2004).

Los suplementos de aceites de semillas vegetales pueden diferir ampliamente en su perfil de ácidos grasos y provocar cambios importantes en la composición de la grasa láctea. La semilla o aceite de lino cuenta con un alto contenido en linolénico (50%) y linoleico (15%), lo que unido a su disponibilidad, lo hace muy adecuado para emplearse en suplementación a la dieta del rumiante (Akraim *et al.*, 2007; Chilliard *et al.*, 2007), habiéndose ensayado ya con éxito en ganado vacuno (Khanal, 2004) y ovino (Luna *et al.*, 2005b). Se ha descrito que el contenido total de CLA en grasa de leche de vaca oscila entre el 0.3-1.0 g/100g grasa (Ritzenthaler *et al.*, 2001), mientras que empleando aceites de soja, canola, girasol o lino los contenidos de CLA pueden llegar a alcanzar valores de 2.13-0.71%, 1.10-0.51%, 2.55-0.72% y 1.70% respectivamente (Kelly *et al.*, 1998; Dhiman *et al.*, 2000; Loor & Herbein, 2003).

Se ha descrito que la inclusión en la dieta del ganado vacuno de maíz y en general, dependiendo de la relación suplemento/forraje, puede producirse un incremento en la concentración de TFA y principalmente del isómero C18:1 t10, que además de las posibles implicaciones de salud por tratarse de un ácido graso *trans*, se ha descrito como inhibidor de la lipogénesis en glándula mamaria, por lo

cual disminuye el contenido graso de la leche producida; efectos similares se han publicado para el isómero de CLA C18:2 t10,c12 (Bauman & Griinari, 2003).

Aunque el ganado vacuno es la principal especie rumiante empleada en la producción de alimentos como carne y lácteos, en los países de la cuenca mediterránea, existe gran interés en la investigación sobre los efectos de la suplementación y mejora del perfil lipídico de la leche de otras especies como cabra y oveja. En la Unión Europea, la producción de leche de cabra representó el 13% del total mundial (del que España representa el 24%), mientras que en leche de oveja esta producción representó el 28% (17%, España) (FAOSTAT, 2004). El hecho de que el manejo de estos animales se produzca en zonas áridas o semiáridas, los hace muy útiles en estos climas, siendo además estas explotaciones respetuosas con el medio ambiente. La investigación en este sentido, puede tener repercusiones a nivel económico, puesto que el sistema de alimentación empleado es responsable del 50-90% del coste por litro de leche producido (Morand-Fehr *et al.*, 2007; Sanz Sampelayo *et al.*, 2007).

Las leches de cabra y oveja presentan mayor viscosidad y acidez pero menor índice de refracción y punto de congelación que la de vaca (Parkash & Jenness, 1968; Juárez & Ramos, 1986; Park, 2008). En cuando a composición de nutrientes, la leche de cabra presenta valores medios similares a los de la leche de vaca en contenido graso (3.8% vs. 3.6%), sólidos no grasos (8.9% vs. 9%), lactosa (4.1%-4.7%), proteínas (3.4% vs. 3.2%), nitrógeno no proteico (0.4% vs. 0.2%) y aporte calórico (70 cal/100 mL vs. 69 cal/100 mL) mientras que la leche de oveja presenta mayor contenido en grasa (7.9%), sólidos no grasos (12%), proteínas (6.2%) y aporte calórico (105 cal/100 mL), mientras que los valores medios para lactosa y nitrógeno no proteico son de 4.9% y 0.8% respectivamente (Larson & Smith, 1974; Posati & Orr, 1976; Jenness, 1980).

La fracción lipídica de la leche de estos animales se caracteriza por encontrarse en forma de glóbulos de un tamaño medio de 3.5 μm, siendo los de mayor diámetro los presentes en leche de oveja (5 μm), seguidos por los de leche de

vaca (4 μm) y por último, los de leche de cabra (2.7-3.5 μm) (Michalski *et al.*, 2004; Lopez, 2005; Park, 2006; Ben Amara-Dali *et al.*, 2008; Martini *et al.*, 2008),
La presencia de aglutininas en la leche bovina hace que tenga una mayor tendencia al desnatado que la leche de cabra que no las contiene (Jennes & Parkash, 1971). A pesar de estos datos, no se han encontrado diferencias en el proceso de secreción y formación de MFGM en ninguna de estas especies de rumiantes (Park *et al.*, 2007).

En cuando a la composición en ácidos grasos en leche de cabra y oveja (Tabla 1) destacan los contenidos en ácidos grasos de cadena corta y media (butírico, caproico, caprílico, cáprico y laurico), que se encuentran en concentraciones superiores a las que pueden encontrarse en leche de vaca (Fontecha *et al.*, 2000; Chilliard *et al.*, 2003; Goudjil *et al.*, 2004). Estos ácidos grasos suelen encontrarse en la posición sn-3 del triglicérido, mientras que los de mayor longitud de cadena (C10-C16), estarán en posición sn-2 (Tziboula-Clarke, 2003). Además el 75% de los ácidos grasos de la grasa láctea de las leches de cabra y oveja corresponde a cáprico, láurico, palmítico, esteárico y oleico (Alonso *et al.*, 1999; Goudjil *et al.*, 2004). El contenido en TFA se encuentra en el rango entre el 5 al 2.5%, con vacénico como ácido graso mayoritario (Park *et al.*, 2007) y las concentraciones de CLA son del 1.1%, 0.7% respectivamente frente al 1% en leche de vaca (Jahreis *et al.*, 1999). Esta composición en ácidos grasos hace que estas leches sean interesantes también desde el punto de vista de la salud por su alta concentración de triglicéridos de cadena media (MCTG) que al ser hidrolizados por la lipasas pueden ser absorbidos sin necesidad de su reesterificación en TG por la células del intestino, entrando directamente al torrente sanguíneo y llegando hasta el hígado y los tejidos (Sanz Sampelayo *et al.*, 2007). Los ácidos grasos de cadena media son digeridos totalmente por las lipasas del estómago mientras que en el caso de ácidos grasos de cadena larga la hidrólisis es parcial y es terminada por la lipasa pancreática. Estos ácidos grasos son empleados por nuestro organismo como fuente rápida de energía por lo que no suelen acumularse en el tejido adiposo (Molkentin, 2000). Las leches de cabra y oveja son por tanto indicadas en personas que presenten insuficiencias pancreáticas o deficiencia o ausencia de sales biliares y en situaciones de desnutrición infantil.

También se han indicado como una alternativa en niños y adultos con intolerancia alérgica a la leche de vaca (Mir *et al.*, 1999).

Al igual que en leche de vaca, la alimentación de estos animales se hace en base a pastos o mediante el empleo de suplementos (Morand-Fehr *et al.*, 2007). Se han desarrollado diversas experiencias para mejorar el perfil lipídico de la grasa de leche de cabra y oveja y conseguir una reducción del contenido en SFA y aumentar los PUFA, habiéndose encontrado que el empleo de fuentes de linoleico y linolénico como aceites y semillas de girasol, soja, lino, etc. producen los mejores resultados (Dhiman *et al.*, 2000; Luna *et al.*, 2005c; Nudda *et al.*, 2006; Luna *et al.*, 2008a; Luna *et al.*, 2008b).

Tsiplakou et al. (2006) describieron el perfil lipídico de leche cabras y ovejas alimentadas mediante pastos en un periodo de Enero a Junio. En ovejas, el contenido en SFA estuvo comprendido entre el 53-48%, MUFA 34-19%, mientras que los PUFA 8.1-5.5%. Las variaciones se debieron a la estacionalidad. En cabras el contenido en SFA fue del 54-59%, MUFA 16-25% y PUFAs (4%) con linoleico (1.8-2.9%) y linolénico (1-0.3%), mientras que las cantidades de CLA estuvieron entre 0.5-0.7%.

Diversos trabajos han estudiado el efecto de la suplementación de la dieta de cabras y ovejas con fuentes lipídicas de alto contenido en linoleico. Bernard et al (2005) compararon el efecto en el perfil de ácidos grasos de la leche de cabra y oveja, de la alimentación de los animales con heno, frente a la adición de un 3.6% de semillas de lino y semillas de lino tratadas con formaldehido para mejorar su digestión. Se observó en cabras alimentadas con semillas tratadas, los mejores resultados en la obtención de grasa láctea más insaturada, con una disminución media del 40% de los SFA frente a la alimentación con heno, mientras que en los MUFA, el oleico tuvo un aumento del 50% en su concentración. En la fracción de PUFA, linoleico no registró variaciones en su concentración mientras que linolénico pasó de un 0.96% en la dieta control a un 2.29% en la dieta suplementada con semillas de lino. En cuanto al CLA (RA fue el único isómero reportado) también aumentó por las dietas suplementadas con semillas, ya que mientras que en la alimentación que únicamente contenía heno, la leche tuvo una concentración de 0.77%, en las dietas que contenían semillas tratadas, los valores fueron de 1.9% y 0.9% con semillas de lino sin tratamiento.

En ovejas los resultados fueron cualitativamente los mismos. Los contenidos en RA fueron positivamente afectados por la suplementación (0.64% en alimentación con heno vs. 0.91% vs. 0.84% en dietas suplementadas con semillas).
Estudios posteriores empleando semillas de lino extrusionadas en cabras (Nudda *et al.*, 2006) y un suplemento comercial a base de lino en ovejas (Luna *et al.*, 2005c) confirman estos resultados. Los ácidos grasos de cadena corta no mostraron variaciones, mientras que el palmítico, que proviene de la síntesis *de novo*, se encontró en menor concentración (35.8% vs. 27.5 en cabras y 28.3% vs. 24.9% en ovejas) y el esteárico, producido en la biohidrogenación en el rumen y en la glándula mamaría por la Δ^9-desaturasa, aumentó por efecto de la suplementación del 6.91% al 10% en cabras y del 8.12% al 10.5% en ovejas. El empleo de dietas suplementadas con fuentes lipídicas poliinsaturadas en la alimentación, también hizo que el contenido en oleico se incrementara un 13% en ovejas y un 26% en cabras y linoleico, 50% de aumento en ambos casos. Estos estudios también reportaron el efecto sobre el contenido en CLA total, dando lugar a resultados de un incrementó del 27% en ovejas y 66% en cabras. Sin embargo, algunos de los estudios mencionados, muestran aumentos inferiores a los de Bernard et al. (2005). Ello puede atribuirse a que la composición de la grasa láctea en rumiantes está condicionada por la genética del animal, su estado de lactación y de salud, por la estación del año y por el tipo de alimentación.

En otros trabajos se han ensayado otras formulaciones empleando aceites de girasol junto con semillas de lino (extrusionadas) de tal forma que se completa la dieta y cuente con alto aporte de linoleico y α-linolénico, principales sustratos para la producción de CLA por las reacciones del rumen/glándula mamaria en cabras y ovejas (Luna *et al.*, 2008a; Luna *et al.*, 2008b)**.** En estos trabajos se encontró que el contenido de todos de los ácidos grasos de 12 a 18 átomos de carbono disminuye, así como la concentración de oleico. El contenido en linoleico en la leche así obtenida aumentó de forma significativa y las cantidades de CLA fueron incrementadas por encima del 50%, siendo RA el principal isómero afectado. Estos estudios ponen de manifiesto la necesidad de que los sustratos de las reacciones de biohidrogenación estén en forma fácilmente accesible para las

enzimas bacterianas, lo cual no sucede cuando en las situaciones en las que se emplean semillas sin tratar.

El elevado número de artículos científicos indica el enorme interés por estudiar la posibilidad de la mejora del perfil lipídico de la leche, a través de la dieta, en estos animales. Sin embargo, la producción de lácteos con grasa más insaturada abre nuevos campos de estudio ante la pregunta de los posibles efectos del procesado en leche y productos lácteos ricos en ácidos grasos poliinsaturados, así como las posibles consecuencias organolépticas del producto elaborado (principalmente queso).

1.2.2 Bacterias productoras de CLA

Como se ha indicado, son las bacterias presentes en el rumen las que llevan a cabo la transformación de los ácidos grasos insaturados de la dieta del rumiante. Este hecho lleva a plantearse si otras bacterias como la empleadas en la fermentación de la leche en la elaboración de yogures y quesos, en presencia de distintos PUFAs, como sustrato, serian capaces de transformarlos en isómeros del linoleico conjugado, siendo por tanto una forma de incrementar los contenidos de estos compuestos en productos lácteos fermentados (Alonso *et al.*, 2003; Van Nieuwenhove *et al.*, 2007). Diversas revisiones (Sieber *et al.*, 2004; Ogawa *et al.*, 2005) han descrito los géneros de bacterias lácticas (Tabla 5) capaces de producir isómeros de CLA en medios donde se adicionan diferentes ácidos grasos insaturados como sustrato, tales como TVA, linoleico, ricinoleico o aceites como el de cártamo con una alta concentración de LA o el de ricino con alto contenido en ricinoleico (C18:1 12-hidroxi-9c). Se ha sugerido que los PUFA son tóxicos para estas bacterias ya que alteran la permeabilidad de la membrana y la transformación en CLA sería, un mecanismo de detoxificación (Nieman, 1954) que incluso pudiera llegar a producir ácidos grasos saturados (Adamczak *et al.*, 2008). Lin *et al.* (1999) encontraron que el crecimiento de varias especies microbianas era inhibido por concentraciones crecientes de linoleico en medios de cultivo, lo que también fue reportado por Wang *et al.* (2007) en leche desnatada. Estos últimos autores, también observaron que la producción de CLA era menor en condiciones de presencia de oxígeno y que tras alcanzarse un máximo en la producción a las 24 horas de iniciada la experiencia, las concentraciones disminuían. Los autores concluyen que reacciones de oxidación o desaturasas bacterianas son la causa de los resultados obtenidos. Otros autores (Ogawa *et al.*, 2001) propusieron que la presencia de oxígeno promovería un metabolismo oxidativo por parte de la bacteria. Cuando el substrato adicionado es un aceite rico en linoleico o ricinoleico, el primer paso por parte de la bacteria es la producción de lipasas que liberen los ácidos grasos del triglicérido (Holland *et al.*, 2005; Di Cagno *et al.*, 2006).

Tabla 5. Producción de CLA por microorganismos.

Cepa	Medio de reacción/Substrato	Isómero de CLA producidos	mg/l
Bifidobacterium aldolescentis	c/LA	cis-9,trans-11 (46%), trans-9,trans-11 (20%), trans-10,cis-12 (34%)	3,5
Bifidobacterium angulatum	c/LA	cis-9,trans-11 (50%), trans-10,cis-12 (50%)	1,2
Bifidobacterium bifidum	c/LA	cis-9,trans-11 (100%)	1
Bifidobacterium breve	c/LA	cis-9,trans-11 (91%), trans-9,trans-11 (9%)	398
Bifidobacterium dentium	c/LA	cis-9,trans-11 (78%), trans-9,trans-11 (21%), trans-10,cis-12 (1%)	160
Bifidobacterium infantis	c/LA	cis-9,trans-11 (74%), trans-9,trans-11 (19%), trans-10,cis-12 (7%)	24,6
Bifidobacterium lactis	c/LA	cis-9,trans-11 (90%), trans-9,trans-11 (8%), trans-10,cis-12 (2%)	170
Bifidobacterium pseudocatenulatum	c/LA	cis-9,trans-11 (72%), trans-9,trans-11 (19%), trans-10,cis-12 (9%)	23,3
Lactobacillus acidophilus	c/LA	cis-9,trans-11 (85%), trans-9,trans-11 (5%), trans-10,cis-12 (10%)	131
Lactobacillus casei	c/LA	cis-9,trans-11 (85%), trans-9,trans-11 (3%), trans-10,cis-12 (12%)	111
Propionibacterium freudenreichii	c/LA	cis-9,trans-11 (93%)	265
L. acidophilus AKU 1137	c/LA	cis-9,trans-11 (57), trans-9,trans-11 (43%)	1500
L. acidophilus IAM 10074	c/LA	cis-9,trans-11 (30%), trans-9,trans-11 (70%)	600
L. acidophilus AKU 1122	c/LA	cis-9,trans-11 (17%), trans-9,trans-11 (83%)	120
L. paracasei ssp. paracasei IFO 12004	c/LA	cis-9,trans-11 (25%), trans-9,trans-11 (75%)	200
L. paracasei ssp. paracasei JCM 1109	c/LA	cis-9,trans-11 (29%), trans-9,trans-11 (71%)	70
L. paracasei ssp. paracasei AKU 1142	c/LA	cis-9,trans-11 (57%), trans-9,trans-11 (43%)	70
L. paracasei ssp. paracasei IFO 3533	c/LA	cis-9,trans-11 (46%), trans-9,trans-11 (44%)	90
L. rhamnosus AKU 1124	c/LA	cis-9,trans-11 (49%), trans-9,trans-11 (51%)	1410
L. brevis IAM 1082	c/LA	cis-9,trans-11 (42%), trans-9,trans-11 (58%)	550
P. shermanii AKU 1254	c/LA	cis-9,trans-11 (82%), trans-9,trans-11 (18%)	110
Enterococcus faecium AKU 1021	c/LA	cis-9,trans-11 (40%), trans-9,trans-11 (60%)	100
L. plantarum	r/CO	cis-9,trans-11 (26%), trans-9,trans-11 (74%)	2700
Butyrivibrio fibrisolvens	r/LA	cis-9,trans-11 (95%)	220
Lactobacillus reuteri	r/LA	cis-9,trans-11 (59%), trans-10,cis-12 (41%)	300
Lactobacillus acidophilus	r/LA	cis-9,trans-11 (67%), trans-9,trans-11 (33%)	4900
Lactobacillus plantarum	r/LA	cis-9,trans-11 (38%), trans-9,trans-11 (62%)	40000
L. plantarum	r/RA	cis-9,trans-11 (21%), trans-9,trans-11 (79%)	2400

LA: ácido linoleico; CO: aceite de colza; c: cultivo; r: resting cells. Datos: Ogaba et al. ,2005, Sieber et al., 2004.

Una vez los substratos se encuentran en su forma libre y en condiciones de microaerobiosis, el ricinoleico es transformado enzimáticamente en linoleico para, tras una reacción de hidratación, dar lugar a la aparición de C18:1 10-hidroxi-12c y C18:1 10-hidroxi-12t a partir de los cuales se forma C18:2 t10,c12 CLA y RA (Ogawa *et al.*, 2001; Kishino *et al.*, 2002b). Otros autores han observado la presencia del isómero C18:2 t9,t11 que se forma por una ruta similar a las anteriores (Alonso *et al.*, 2003; Ando *et al.*, 2003; Bisig *et al.*, 2007).

También se han reportado aumentos en las concentraciones de los ácidos TVA y esteárico por parte de bacterias del género *Lactobacillus* y *Bifidobacterium* (Xu et al., 2008), lo que indicaría que la transformación de LA en CLA no se produciría a través de las reacciones descritas y que conducen a la formación de hidroxiácidos, si no por biohidrogenación. Tales diferencias permiten pensar que en un metabolismo oxidativo la producción de CLA se produce a través de reacciones de hidratación, mientras que en los casos donde este metabolismo no se activa no es geneticamente posible realizarlo, la producción es mediante biohidogenación, a semejanza de lo que acontece en el rumen.

En algunos trabajos se exponen los resultados de experimentos en los que no se encontró una producción significativa de CLA en sistemas binarios de microorganismos (*S. thermophilus* con *L. acidophilus, L. bulgaricus*, *L. rhamnosus* y *B. lactis*) en leche desnatada sin adición de ninguna fuente lipídica (Oliveira *et al.*, 2009). Otros estudios en donde se emplearon bacterias probióticas como *L. acidophilus* o *B. animalis* junto con un cultivo iniciador (*S. thermophilus and L. delbreckii ssp. bulgaricus*) y adicionando a la leche fructooligosacaridos (FOS) para que actuaran como prebióticos en la flora intestinal del consumidor, observaron contenidos de CLA en las muestras control (yogur fermentado con el cultivo iniciador y sin FOS) de 2 mg/ g grasa, mientras que en los yogures con bacterias probioticas y FOS, la concentración de CLA fue (en el caso de *B. animalis*) de 6 mg CLA/g grasa (Akalin *et al.*, 2007).

Puniya *et al.* (2008) encontraron que *L. brevis* aislado de fluidos ruminales producía 10 mg CLA/g grasa en leche desnatada usando aceite de girasol (0.25%) como fuente de linoleico, mientras que *L. lactis* con 1% de aceite de girasol produjo 9.22 mg CLA/g grasa. En otras investigaciones (Xu *et al.*, 2004) llevadas a cabo con *P. freudenreichii*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L.*

rhamnosus, E. faecium, *P. acidilactici,* y *B. bifidum* en leche desnatada con aceite de soja hidrolizado (1% de contenido graso total), las concentraciones de CLA encontradas fueron de 2.21-0.63 mg/g grasa. Cuando la fuente lipídica fue grasa láctea, solamente *P. freudenreichii*, *E. faecium, P. acidilactici* mostraron capacidad de producir isómeros de CLA en concentraciones de 0.2 mg/g grasa. Otras investigaciones mostraron como en leche de búfala pasteurizada y en presencia de las bacterias *L. acidophilus, L. casei* y *Lc. Lactis*, producían 6 mg CLA/g grasa a partir de grasa láctea (Yadav *et al.*, 2007).

Otros autores han llevado a cabo experiencias con lo que se ha denominado como células lavadas, "*washed cells"*, bajo el concepto de usar a los microorganismos como catalizadores de la reacción. Las bacterias, tras un periodo de crecimiento/acondicionamiento en presencia de ácido linoleico, ricinoleico o aceites que los contengan, son aisladas y transferidas a un medio de reacción en donde la concentración de substrato es mayor. En estas condiciones, Ando *et al.* (2003), empleando *Lactobacillus, Streptococcus y Pediococcus* y ricinoleico como sustrato reportó que las concentraciones de CLA producidas variaron de los 0.14 a los 1.1 mg/ mL de medio de reacción.

Estudios de Kishino *et al.* (2002a) empleando linoleico en el crecimiento de *L. plantarum* y ácido ricinoleico, ésteres metílicos de LA y aceite de ricino en la mezcla de reacción encontraron que únicamente se produjo síntesis de CLA cuando se emplearon ácidos grasos libres (1.13-1.65 mg/mL en medio de reacción con ricinoleico y aceite de ricino tratado con lipasas respectivamente). En otros trabajos de investigación, también con *L. plantarum*, este mismo grupo obtuvo producciones de 3,88 mg CLA/ mL de medio de reacción, usando linoleico libre, tanto en el crecimiento, como en el medio de reacción (Kishino *et al.*, 2002b).

Recientemente este grupo de investigación (Kishino *et al.*, 2009) ha reportado que células lavadas de *L. plantarum*, son capaces de transformar, a las 48h de incubación a 37ºC en microaerobiosis, el ácido α-linolénico (0.3% en el medio de reacción) en isómeros de CLnA, que representaron el 41.7% del total de los ácidos grasos encontrados.

1.2.3 Derivados lácteos suplementados/enriquecidos en PUFA

La estabilidad y biodisponibilidad de los diferentes nutrientes y compuestos bioactivos presentes en los alimentos preparados o formulaciones alimentarias es un tema de especial interés en la ciencia y tecnología de los alimentos. Son numerosos los estudios científicos que han determinado y descrito como se afectan los diferentes componentes de un alimento como consecuencia de los procesos industriales, la manipulación y el posterior almacenamiento a los que son sometidos.

En el caso de alimentos funcionales, enriquecidos, suplementados y/o fortificados en ciertos componentes con actividad biológica, el compromiso del fabricante debe ser garantizar la estabilidad de estos compuestos cuando se adicionan a la matriz alimentaria, durante los procesos tecnológicos y de conservación, almacenamiento durante su vida útil, etc., para asegurar la cantidad y el estado en que ese nutriente bioactivo llega al consumidor.

La estabilidad de los diferentes componentes de un alimento va a depender fundamentalmente de las condiciones y del método del procesado y temperatura de conservación. Así las vitaminas A, B1, B12, C y D, son consideradas las menos estables y tienden a ser más susceptibles a la oxidación en presencia de iones metálicos o acidez del medio (pH menor de 5). Algunos minerales adicionados se caracterizan por formar complejos que generan problemas de solubilidad en los sistemas alimentarios.
De la fracción lipídica de un alimento son principalmente sus ácidos grasos poliinsaturados, los componentes más sensibles a los procesos industriales. El tiempo de exposición a distintos factores perjudiciales (temperatura, luz, oxígeno) produce una reducción importante en su contenido debido al desarrollo de procesos oxidativos con la posterior aparición de sabores y olores desagradables.

En el mercado actual existen numerosos derivados lácteos en donde la grasa láctea ha sido reemplazada o adicionada con aceites vegetales y/o de pescado y

en cuya composición se encuentran ácidos grasos con contrastado efecto positivo sobre la salud.

1.2.3.1 Derivados lácteos suplementados con CLA

El contenido en CLA en lácteos y carne varía entre 1,07-0,34 % y 0,68-0,12 % de la grasa total respectivamente, por lo que se ha calculado que la ingesta media diaria de CLA en la dieta occidental es de 200-100 mg (Ritzenthaler *et al.*, 2001), y su consumo se sigue reduciendo como consecuencia de la disminución de la ingestión de leche entera, mantequilla y carne de rumiantes. En España según datos del MAPA (2006), la ingesta estimada de CLA es de 112.5 mg/persona/día. Este valor es bastante moderado e inferior a los niveles recomendados para resultar efectivo en alimentación humana (Williams, 2000) y muy por debajo de las dosis utilizadas en los estudios con animales de experimentación y que obtienen efectos en la reducción de ciertos tipos de tumores. Si tenemos en cuenta que los experimentos llevados a cabo en animales y extrapolados a humanos, indican que para obtener los efectos biológicos del CLA, se necesitaría una dosis de 3g CLA/día (Ip *et al.*, 1994), el consumo de derivados lácteos y carne de rumiantes necesitaría ser muy elevado.

Por ello, se están realizando esfuerzos en la línea de diseñar estrategias encaminadas a incrementar los contenidos de estos isómeros de CLA (fundamentalmente RA y C18:2 t10,c12*)* en leches de consumo y productos lácteos, ya que sin duda pueden repercutir de forma potencialmente favorable en su calidad nutricional y terapéutica y por supuesto en el valor añadido de los alimentos que los contengan.

Mezclas de isómeros de CLA se producen de forma sintética mediante isomerización alcalina de aceites ricos en ácido linoleico (cártamo y girasol fundamentalmente) y se encuentran disponibles comercialmente en forma de cápsulas de gelatina desde 1995 en EEUU y más recientemente en Europa y Japón. Estos preparados de CLA, que contienen predominantemente los isómeros C18:2 c9,t11 y C18:2 t10,c12, aproximadamente al 80% y repartidos en proporción similar (1:1) (Ma *et al.*, 1999), han sido ampliamente utilizados en estudios científicos para determinar y comprobar sus efectos biológicos. Esta

mezcla de los dos isómeros resultan más eficaces y seguras que otras según los estudios clínicos realizados (Gaullier *et al.*, 2002). Actualmente existen en el mercado español derivados lácteos a los cuales, tras el desnatado de la leche se les adiciona un aceite comercial (Tonalin®) que presenta esta composición de isómeros de CLA donde RA y C18:2 10t, 12c están en proporción 1:1 y representan el 80% del total de ácidos grasos. La gama de productos incluye leche (1% contenido graso), yogures cremosos (1.6%) y líquidos (1%). En ellos, el fabricante declara que una dosis diaria (250 mL en el caso de la leche, 1 envase en los yogures) contiene la cantidad necesaria de CLA necesaria para producir sus efectos biológicos.

1.2.3.2 Derivados lácteos de alto contenido en PUFAs.

Desde un punto de vista nutricional, la dieta de la sociedad industrializada actual se caracteriza por un consumo energético elevado, asociada al incremento de la ingesta de ácidos grasos saturados, *trans* y de aceites de semillas (ricos en linoleico, ω6), así como por una disminución del consumo de ácidos grasos ω3 (presentes en pescados grasos), frutas, verduras, carbohidratos complejos y fibra. Todo ello conduce a un aumento de las enfermedades asociadas a los patrones alimentarios, como las enfermedades cardiovasculares y el síndrome metabólico. Las enfermedades relacionadas con el sistema cardiaco causan al año 1.5 millones de muertes en la UE mientras que en términos económicos, se ha estimado que suponen una gasto para los sistemas sanitarios de 230€ por ciudadano, ascendiendo el gasto global a 168 billones de euros al año (Leal *et al.*, 2006).

En el cuerpo humano, el ácido palmítico y esteárico son los precursores de los ácidos grasos monoinsaturados mayoritarios en nuestras células: por desaturación el C16:0 es convertido en ácido palmitoleico (C16:1 c9), mientras que otras rutas de elongación y desaturación dan lugar a la aparición de esteárico, oleico, ácidos grasos C18:2 y otros de mayor longitud de cadena (C20:1, C22:1 y C20:2) (García Muriana, 2002). Sin embargo, nuestras células no producen las desaturasas capaces de introducir dobles enlaces en las posiciones

12 y 15, por lo que los ácidos grasos de la familia omega 6 y 3 son esenciales en el ser humano: El ácido linoleico es el precursor de araquidónico, mientras que α-linolénico lo es de EPA, DPA y DHA (Williams, 2000).

Los ácidos grasos poliinsaturados ω6 y ω3 son almacenados en los fosfolípidos de las membranas de las células y su hidrólisis se produce mediante la acción de fosfolipasas (A_2). Estas enzimas que actúan en la posición 2 del fosfolípido conducen a la liberación, fundamentalmente de ácido araquidónico, el cual está implicado en las rutas de síntesis de prostaglandinas, leucotrienos y tromboxanos relacionados con los procesos inflamatorios (Calder, 2006). Los ácidos grasos la familia ω3 pueden inhibir tanto la síntesis como la acción de las fosfolipasas A_2. Diversos estudios demuestran que DHA, EPA y DPA tienen efectos beneficiosos sobre los niveles de colesterol y triglicéridos en sangre (Berry *et al.*, 1991; Griffin *et al.*, 2006; Warensjo *et al.*, 2006) así como efectos antiinfalmatorios y de agregración plaquetaria (Calder, 2006).

Sin embargo, el ácido linoleico es competidor de las rutas de síntesis de ácidos grasos ω3, lo que en dietas con un alto consumo de aceites vegetales de semillas y bajo en pescado, como las que se producen en países industrializados, conduce a un incremento del riesgo de enfermedades cardiovasculares. De ahí la importancia del balance ω6/ω3 en la dieta, el cual debiera estar, en un rango de 5-10/1, mientras que actualmente se encuentra en 15-20/1 (Stone, 1996; De Lorgeril *et al.*, 1997; Connor, 2001).

Recientemente la EFSA ha declarado que la ingesta de EPA y DHA diaria debiera ser en Europa de 250 mg, 2 g para linolénico y 6 g para linoleico. No obstante, datos de el consumo de estos ácidos grasos indican que la ingesta de estos ácidos grasos es <100 mg/día de EPA+DHA, 0.7-2.3 g/dia de linolénico y 7-19 g/dia de linoleico (Ian Givens & Gibbs, 2008; EFSA, 2009).

Actualmente en el mercado existen diversos productos lácteos desnatados o semidesnatados (leche y productos fermentados) en donde se han añadido aceites con unos contenidos totales de DHA y EPA en un rango de 60-37 mg/100 g de producto.

Los aceites de soja se caracterizan por tener contenidos de linolénico del 8% y de linoleico del 50%. Otras marcas optan por adicionar estos aceites a sus derivados lácteos (leche y yogures) con lo que la relación ω6/ω3 seria de aproximadamente 10:1.

1.2.3.3 Fórmulas infantiles.

La ESPGHAN, sociedad europea para la pediatría, gastroenterología, hepatología y nutrición declara que la alimentación infantil de lactantes en las denominadas leches de inicio (4-6 primeros meses de vida), debe cumplir con un aporte lipídico de 4.4-6.0 g/100 Kcal (64-70 Kcal/100 mL), de los que linoleico representará 500-1.200 mg/100 Kcal y linolénico >55 mg/100 Kcal. Para las leches de continuación (lactantes de 5-12 meses y niños entre 1-3 años) además se especifica que el contenido en láurico y mirístico deben ser inferior al 15% del total de ácidos grasos (ESPGAN, 1982).

En la preparación de la mayoría de estos alimentos, la leche de vaca (al ser muy similar a la humana) es desnatada y la fracción lipídica sustituida por una mezcla de aceites vegetales, de pescado y otros (obtenidos de hongos, algas, etc.) para que la composición grasa sea similar a la que se encuentra en la leche materna y los contenidos en ácidos grasos de cadena larga sean los requeridos en estas etapas de desarrollo .

Los datos acumulados de estudios clínicos y científicos sobre ácidos grasos poliinsaturados de cadena larga (LCPUFAs), como DHA y AA, empleados como aditivos para las fórmulas infantiles sugieren que estos son esenciales para el aporte energético, el crecimiento, la diferenciación de órganos y el metabolismo celular. Estos ácidos grasos son importantes para el desarrollo del sistema nervioso central y cognitivo de los niños en su etapa de crecimiento. Diversos estudios demuestran que el AA es un componente estructural de los tejidos del cerebro, mientras que el DHA que se encuentras en altas concentraciones en la retina, está implicado en el desarrollo de las conexiones entre neuronas (Jensen

et al., 1990; Innis, 1991; Martinez, 1992; Hornstra, 2000; Agostoni & Giovannini, 2001; Gil *et al.*, 2003).

En una revisión llevada a cabo en el presente trabajo, de los productos presentes en el mercado español, existen diversas marcas que comercializan fórmulas infantiles como preparados en polvo o en forma líquida con estos LCPUFAs adicionados. El aporte energético se encuentra entre las 60-80 Kcal/100 mL (3.3-6.5 g grasa/100 Kcal) en donde la composición en ácidos grasos tiene un contenido medio en linoleico de 400 mg/ 100 Kcal, 300mg/100 Kcal de linolénico, 11 mg/ 100 Kcal de láurico y 4.9 mg/100 Kcal de mirístico.

1.3 PROCESADO Y CONSERVACIÓN DE LACTEOS Y DERIVADOS.

1.3.1 Efecto del procesado de productos lácteos en los ácidos grasos

En la elaboración de productos lácteos y derivados, la legislación obliga al empleo de tratamientos térmicos tales como la pasterización, esterilización o UHT, para lograr la seguridad microbiológica y prolongar la vida útil del producto. Sin embargo estos procesos pueden generar sabores extraños y alterar las características sensoriales del alimento (Smiddy et al., 2007).

Los tratamientos de pasteurización (HTST, alta temperatura corto tiempo) a 72 °C durante 15 s imparten un ligero sabor a cocido (sulfuroso) que ha pasado a ser aceptable por los consumidores de leche, pero su vida útil en refrigeración es de sólo 15-20 días. Otros procesos térmicos como la ultra-pasteurización (UP) a distintas temperaturas y tiempos (e.j. 1s a 89ºC, 0.1s a 96ºC ó 0.01s a 100ºC) o de temperatura ultra alta (UHT) de procesamiento a 135-150°C durante 3-5 s, permiten alargar aun más la estabilidad de la leche hasta 3-6 meses a temperatura ambiente. Sin embargo estos procesos inducen fuertes sabores a "cocido" (Colahan-Sederstrom & Peterson, 2005). Numerosos estudios han identificado compuestos volátiles sulfurados, aldehídos y cetonas como importantes contribuyentes a los “off-flavors” encontrados en la leche (Contarini & Povolo, 2002; Vazquez-Landaverde *et al.*, 2006).

La grasa láctea es relativamente estable frente a los tratamientos térmicos, aunque en condiciones de elevada temperatura y presencia de oxígeno y metales, puede condicionar la oxidación y degradación de los ácidos grasos poliinsaturados, dando lugar a la aparición de compuestos polares de potencial toxicidad. Los ácidos grasos insaturados, especialmente aquellos con dos o más dobles enlaces son sensibles a la luz, la temperatura y el oxígeno. Por otra parte, los productos de oxidación de los lípidos no sólo causan rancidez, sino que provocan alteraciones en los aminoácidos, proteínas y otros componentes de los alimentos.

Sin embargo, y aunque la fracción lipídica, y en particular los ácidos mono y poliinsaturados son igualmente susceptibles a cambios inducidos por los

tratamientos térmicos de uso común en la industria láctea, estos procesos no han sido estudiados en profundidad en derivados lácteos cuando su grasa ha sido sustituida o modificada (Jensen, 2002).

Giroux *et al.* (2008) y Schreyer *et al*. (2008) observaron que en leches tratadas por pasterización el potencial redox es menor y la concentración de oxígeno era mayor que en los tratamientos de UHT o de esterilización, dando lugar a entornos más oxidantes. Por otra parte, los tratamientos térmicos producen desnaturalización de proteínas que actuarían como donadores de protones e inactivarían especies químicas sensibles a la oxidación, mientras que las altas temperaturas favorecen la aparición de reacciones de Maillard, teniendo algunos de sus compuestos, en los que se encuentren presentes grupos sulfidrilo, capacidades antioxidantes. Se ha descrito que el tratamiento térmico de leche desnatada provoca un aumento de los grupos reactivos sulfidrilo en las caseinas, aumentando la capacidad antioxidante de la leche (Taylor & Richardson, 1980), pero también la aparición de productos de oxidación (grupos carboxilo) (Fenaille *et al.*, 2006). Además de que los compuestos de Maillard pueden interaccionar con los lípidos dando lugar a la aparición de compuestos volátiles (Whitfield, 1992) e igualmente productos de la degradación oxidativa de los ácidos grasos (malondialdehido) pueden reaccionar con proteínas lácteas como las caseínas y provocar la formación de compuestos volátiles (Adams *et al.*, 2008).
Calligaris *et al*. (2004) describieron en leche desnatada pasterizada comercial y tratada posteriormente a temperaturas de 80, 90 y 120ºC durante 24 horas, que la capacidad antioxidante disminuyó como consecuencia de la degradación de los antioxidantes (tocoferoles) de forma inversamente proporcional a la temperatura, al igual que el potencial redox y la actividad prooxidante.

Estudios previos (Precht *et al.*, 1999) llevados a cabo en mantequillas tratadas a temperaturas de 200-300ºC, 15 minutos, mostraron que el ácido oleico podía isomerizar en su compuesto *trans* C18:1 t9. También fueron descritas disminuciones en el contenido de linoleico y linolénico, atribuidos a la formación de compuestos de oxidación.

Destaillats *et al.* (2005) en sistemas modelo, donde un éster metílico puro de linoleico fue tratado térmicamente a temperaturas de 200 y 240ºC hasta un tiempo de 240 horas en presencia o no de oxígeno, observaron que la formación de CLA estaba favorecida por la temperatura y la presencia de oxígeno. Los autores propusieron dos mecanismos para la formación de isómeros de CLA, uno mediado por radicales, en situaciones de alta concentración de linoleico (30-40%), mientras que con contenidos inferiores la reacción se produciría por isomerización del linoleico. En ambos casos los productos finales serían dos isómeros concretos: RA y C18:2 t10,c12. Previamente Juaneda *et al.* (2003), describieron aumentos del contenido isómeros de CLA, RA y C18:2 t10,12c en aceites de girasol calentados a 180 y 220ºC durante 30 minutos.

En otros estudios (Destaillats *et al.*, 2005a) en mantequillas sometidas a 200ºC hasta 6 horas, reportaron que tras tratamientos de 4 horas, se producían reacciones de reordenamiento sigmatrópico dentro de la fracción de CLA. Estas reacciones conllevan el desplazamiento del doble enlace hacia la posición del carbono siguiente o posterior e inversión de la geometría del enlace, por ejemplo C18:2 c9,t11 en C18:2 t8,c10, siendo este el principal cambio observado por los autores, pero también del incremento de los isómeros *trans, trans.*

Herzallah *et al*. (2005b) realizaron estudios donde la leche fue tratada mediante distintos procesos térmicos de pasterización, UHT, microondas y productos fermentados como yogur y queso. Como resultado del procesado térmico por pasterización y microondas, el contenido total de ácidos grasos *trans* aumentó significativamente en comparación con la leche cruda. Observaron además que el contenido en CLA total disminuyó por estos tratamientos y el efecto se atribuyó a reacciones de oxidación. En el estudio se atribuyen los resultados observados en leche pasterizada a que este tipo de tratamientos provocan una mayor concentración de oxígeno en la leche. En el procesado por microondas los efectos producidos se atribuyen a la aplicación de este tipo de energía. La disminución del total de isómeros de CLA concuerda con los resultados expuestos por Campbell *et al*.(2003) al estudiar el efecto de la pasteurización HTST de leche enriquecida con un aceite de alto contenido en CLA. Observaron que el contenido del isómero

RA disminuyó tras el tratamiento y conservación en refrigeración, aunque lo atribuyen a defectos en la calidad microbiológica de la leche.

Lepri et al. (1997) reportan que en leche humana pasterizada, la actividad lipolítica aumentaba tras el procesado, indicado por el aumento en el contenido en ácidos grasos libres. Se ha descrito que aunque los tratamientos térmicos pueden producir hidrólisis de ácidos grasos, en muchos casos son las lipasas microbianas las que se mantienen activas aún después de procesados severos como UHT (Panfil-Kuncewicz *et al.*, 2005b), mientras que la lipasa nativa de la leche quedaría inactiva. Otros estudios llegan a la conclusión de que el procesado térmico no tiene ningún efecto en la composición de los ácidos grasos. Así, Herzallah *et al* (2005a), no encontraron variaciones en los contenidos totales de ácidos grasos saturados, monoinsaturados y poliinsaturados en leche cruda sometida a pasterización, UHT y microondas. Por su parte Jones *et al*.(2005), compararon el efecto del procesado UHT frente a leche cruda con una composición en ácidos grasos mejorada con respecto a los contenidos de trans TVA y CLA y leches de composición normal. El resultado de los estudios muestra que el tratamiento no alteró los contenidos en ácidos grasos, ni la composición de las muestras analizadas. Estos resultados coinciden con los obtenidos por Lynch *et al*.(2005) para leche semidesnatada pasterizada, en donde igualmente los niveles de *TVA* y CLA habían sido incrementados por la suplementación de la dieta con aceites de pescado y soja y no se observaron cambios en la composición en ácidos grasos tras la pasterización HTST.

Sin embargo, otros estudios como los llevado a cabo por Aneja y Murthi (1990) concluyen que el procesado influye en los contenidos de CLA en el producto final, al encontrar contenidos mayores en yogur dahi (producto fermentado hindú) que frente a la leche de partida. Los mismos resultados fueron obtenidos por Lin *et al*. (2003) en yogur debido al efecto del *Lactobacillus acidophilus* empleado en la fermentación. No obstante, Shantha *et al*. (1995) no pudieron reproducir estos resultados en experiencias posteriores durante la producción de yogures y quesos.

Ha *et al*. (1989) reportaron incrementos de CLA en queso (parmesano, cheddar, azul y queso para untar) referidos como resultado de la oxidación del linoleico con la participación de donadores de protones, como las proteínas y que

coinciden con estudios previos (Shantha *et al.*, 1992) también en quesos. Sin embargo, recientes investigaciones realizadas en otros tipos de quesos madurados (Mahón, Manchego y Cabrales) no se han encontrado ni aumentos ni modificaciones significativas en los contenidos de CLA (Luna *et al.*, 2005a; Luna *et al.*, 2007).

1.3.1.1 Nuevas tecnologías de procesamiento: Las altas presiones.

La tendencia creciente de la industria láctea a aumentar el contenido de ácidos grasos poliinsaturados en la leche, presenta la contrapartida de la susceptibilidad de estos compuestos a cambios oxidativos y alteraciones por procesos térmicos. Esto ha conducido a la búsqueda y desarrollo de nuevos procesos tecnológicos e industriales en donde la calidad organoléptica y físico-química, sea lo más parecida al producto fresco.

El procesado de los alimentos a altas presiones hidrostáticas (APH) consiste en la aplicación de presiones muy elevadas (100-1000 MPa) sobre el alimento envasado en un material flexible, en el interior de un cilindro de presurización, durante periodos de tiempo que generalmente se encuentran entre 1 y 30 minutos a temperaturas entre 20 y 60º.

Durante la presurización tiene lugar un aumento de temperatura debido al calentamiento adiabático, que oscila entre 3 y 8ºC por cada 100 MPa. Las instalaciones para la aplicación de APH constan básicamente de una cámara cilíndrica de tratamiento de acero inoxidable; un fluido para transmitir la presión, habitualmente agua, y una bomba para generar la presión. Los equipos utilizados actualmente en la industria pueden tratar hasta 350 litros por ciclo, lo que ha aumentado la capacidad de procesado. El tratamiento por APH es generalmente un proceso discontinuo por lotes, aunque se puede convertir en un proceso semi-continuo si se instalan 3 o más cámaras de tratamiento en paralelo.

Una de las ventajas de la aplicación de APH es que todo el alimento se somete a la misma presión durante el mismo tiempo, a diferencia de lo que ocurre con otras tecnologías, como el tratamiento térmico, en la que se establecen gradientes de intensidad.

El tratamiento de altas presiones y últimamente la homogenización a alta presión (aplicación de altas presiones en los procesos de homogenización) que aúna en un mismo procedimiento la reducción del tamaño de glóbulo graso de la homogenización convencional, con las capacidades antimicrobiológicas de los tratamientos de las altas presiones, permiten obtener productos sanitariamente seguros sin alterar las características sensoriales (Huppertz *et al.*, 2006; Lopez-Fandino, 2006; Serra *et al.*, 2008).

En general, las bacterias, los mohos y las levaduras se inactivan de forma eficaz con tratamientos de intensidad y duración medios; las bacterias Gram-positivas son más resistentes que las Gram-negativas, aunque existen importantes variaciones (Patterson, 2005). Los mohos y las levaduras son relativamente sensibles a esta tecnología mientras que las endoesporas bacterianas como se ha indicado son resistentes, incluso a presiones muy altas. Sin embargo, presiones relativamente bajas, de entre 60 y 100 MPa, inducen la germinación, y con ello la sensibilización de las esporas, por lo que se ha propuesto un tratamiento en varias etapas para mejorar el efecto letal de las APH. Este tratamiento, no obstante, tiene una eficacia variable y depende de los constituyentes del medio. Además, parece que siempre una pequeña proporción de la población de esporas puede permanecer sin germinar. Estudios sobre inactivación microbiana en algunos productos líquidos como la leche han puesto de manifiesto que incluso a presiones en torno a los 1000 MPa no es posible obtener productos estériles. Sin embargo, se puede reducir significativamente la presencia de bacterias psicrótrofas en leche cruda mediante tratamientos a 400 MPa durante 30 min., lo que permite aumentar considerablemente el periodo de conservación en refrigeración.

Por otro lado la puesta en escena de fuerzas de cavitación, fricción, turbulencia y cizalla afectan a las propiedades reológicas del alimento, tanto por la desnaturalización de proteínas del suero y caseínas, como por la rotura de la membrana de los glóbulos grasos (MFGM). La composición de la MFGM quedará alterada al recomponerse, puesto que las proteínas que se han desnaturalizado pueden ahora entrar a formar parte de ella (Michalski & Januel, 2006; Patazca *et al.*, 2007; Zamora *et al.*, 2007).

La bibliografía existente en este tema, es extensa en cuanto a los efectos de las altas presiones en las proteínas lácteas, pero prácticamente inexistente en lo referente al efecto sobre la fracción lipídica. Ello abre un campo a ser estudiado, ante las evidencias de que la reducción en los diámetros del glóbulo graso, unido a la resistencia de la lipasa nativa de la leche al proceso, incrementan el riesgo de lipolisis (Datta *et al.*, 2005; Hayes *et al.*, 2005) y a que ciertos autores han descrito la presencia de sabores extraños atribuidos a oxidación, en condiciones de presión de 40-60 MPa (Humbert *et al.*, 1980; Michalski & Januel, 2006).

1.3.2 Efectos de las condiciones y tiempo de conservación en la fracción lipídica de productos lácteos y derivados

Como se ha expuesto hasta ahora, existe un gran interés en la elaboración de alimentos en los que la composición, permita al consumidor mantener o mejorar su estado de salud. En los productos lácteos y derivados, parte del interés se centra en la mejora de la composición lipídica y principalmente en el aumento de la concentración de ácidos grasos monoinsaturados y poliinsaturados. Estos compuestos son sensibles a alteraciones oxidativas en donde los tratamientos térmicos, como se ha visto anteriormente, junto con iniciadores de reacciones mediadas por radicales, como la luz, el oxígeno o la presencia de metales, conducen a un deterioro en las características sensoriales del alimento, así como pérdida de la calidad nutricional del mismo.

Ciertas etapas del procesado del alimento van a influir en los cambios que tienen lugar durante su conservación posterior. Actualmente, tanto el consumidor como el fabricante buscan comprar/elaborar alimentos cuya vida útil sea lo más extensa posible, lo que en parte favorece el desarrollo de los procesos de degradación. No obstante, como se ha indicado anteriormente, no son muchos los estudios presentes en la bibliografía relacionados con la investigación de la fracción lipídica de los alimentos y en particular de los productos lácteos y derivados tras el procesamiento térmico y/o elaboración y durante su posterior conservación. Así, tanto Herzallah *et al*. (2005a) en productos lácteos (leches pasterizada, UHT,

calentada por microondas, yogures y queso) como Lynch *et al*. (2005) en leche pasterizada donde la alimentación del ganado condujo a mayores contenidos en PUFAs, como Let *et al*. (2005) en derivados lácteos pasterizados enriquecidos con aceites de pescado, no encontraron modificaciones significativas en el total de ácidos grasos libres saturados, monoinsaturados y poliinsaturados durante el periodo de conservación en refrigeración. En otros trabajos de este mismo grupo de investigación (Let *et al.*, 2004) se observaron variaciones en el índice de peróxidos y en el perfil de volátiles en leches enriquecidas con aceites de pescado, atribuidas a que las condiciones de almacenamiento estarían afectando a la fracción lipídica. Otros estudios (Herzallah *et al.*, 2005b) muestran una clara relación entre el tratamiento térmico de leche por pasterización y UHT, así como en la elaboración de yogures con una disminución del contenido total de CLA durante el periodo de conservación en refrigeración.

Chavez-Servin *et al.* (2008) llevaron a cabo estudios sobre el posible efecto del periodo de conservación (70 días) a temperatura ambiente en el perfil lipídico de fórmulas infantiles comerciales en polvo, simulando una situación de empleo doméstico. Observaron perdidas en los contenidos de linoleico y linolénico pero no en otros con mayor grado de insaturación como araquidónico o DHA; la degradación de los ácidos grasos conduce a la aparición de compuestos volátiles, en el caso de compuestos poliinsaturados, hexanal y heptanal, que fueron detectados en algunas de las muestras estudiadas. Las fórmulas infantiles incluyen en su composición ácidos grasos poliinsaturados esenciales, los productos comerciales cuentan con formulaciones (inclusión de antioxidantes) y envases diseñados para la evitar la degradación oxidativa de los ácidos grasos, así como su exposición a la luz y la permeabilidad al oxígeno. No obstante, se han descrito en numerosos estudios científicos las alteraciones observadas en fórmulas infantiles, que en muchos casos se deben a defectos en el procesado pero en general a la inapropiada conservación del producto (Calvo *et al.*, 2003).
Estudios del efecto de la conservación en refrigeración durante 45 días de leche y yogures pasterizados enriquecidos por la adición de aceites de pescado, se encontró un aumento en la concentración de compuestos volátiles producidos por la degradación de hidroperóxidos (Let *et al.*, 2007). En las muestras también se

observó que el índice de peróxidos aumentaba de forma continua durante el estudio, a la vez que la concentración en tocoferoles disminuía, indicando el desarrollo de reacciones de oxidación de la fracción lipídica durante el periodo de conservación en refrigeración.

Un estudio comparativo del efecto del almacenamiento en refrigeración de mantequilla tradicional con otras con un perfil lipídico rico en ácidos grasos insaturados y CLA (Mallia *et al.*, 2008), reportaron que se producían alteraciones del perfil lipídico debido a pérdidas de elaídico (C18:1 t9) y C18:1 t12, mientras que el CLA permaneció estable durante las 8 semanas que duró el seguimiento. Sin embargo, los compuestos responsables del aroma (aldehídos, cetonas y compuestos hidrocarbonados derivados de oxidación lipídica) aumentaron durante la conservación.

Sørensen et al (2007), en estudios para determinar el efecto de la conservación en congelación (15 días, -40ºC) en leches enriquecidas con aceites de pescado pasterizadas y con diferentes condiciones de homogenización (50ºC, 72ºC; 5, 15, 25 MPa), observaron un incremento en el índice de peróxidos en todas las muestras durante el periodo de estudio y que fue mayor en aquellas en las temperatura de homogenización fue más baja. En las leches que fueron tratadas a esta temperatura y a una presión de 5 MPa, la concentración de grupos tiol aumentaron significativamente. Estos resultados muestran claramente que a pesar de las bajas temperaturas de conservación empleadas, tuvieron lugar reacciones de oxidación tanto de lípidos como de proteínas.

Un factor importante en el deterioro de los ácidos grasos durante el periodo de conservación es la calidad microbiológica de la leche. La oxidación puede tener inicio tras la liberación de los ácidos grasos libres por parte de lipasas. Aunque la leche cuenta con una lipasa nativa, esta queda desnaturalizada en los procesos térmicos de pasteurización. Sin embargo, se ha descrito que determinadas lipasas bacterianas de microorganismos psicrótrofos, pueden continuar activas después de tratamientos (UHT y esterilización), por lo que aumentarían la concentración de ácidos grasos libres (ácidos grasos de cadena corta, media, esteárico y oleico) en leche conservada a temperatura ambiente o en refrigeración (Choi & Jeon, 1993;

Panfil-Kuncewicz *et al.*, 2005a). Shantha *et al.* (1995) en yogures conservados a 4ºC, 6 semanas donde se encontró disminuciones del contenido en CLA total y Ruménico. Otros estudios (Kumar *et al.*, 2006) llevados a cabo en leche enriquecida en CLA encontraron que tras pasterizar el producto, el contenido en RA disminuyó a las tres semanas de conservarse en refrigeración e igualmente en la maduración de queso Cheddar, a partir de los 3 meses. Otros trabajos de investigación (Serra *et al.*, 2008) compararon la evolución del contenido en ácidos grasos libres y la posible presencia de compuestos de oxidación lipídica (TBARS) en yogures elaborados mediante ultrahomogenización a altas presiones (UHPH) frente a pasteurizados durante la conservación (15 días), no encontraron ninguna diferencia o variación, a pesar de que las muestras tratadas térmicamente mostraban valores TBARS mayores que las procesadas por UHPH. Esto puede deberse, según los autores, a que los tratamientos térmicos, a pesar de poder producir especies reactivas, también inducían la aparición de grupos tiol en proteínas, que poseen capacidad antioxidante.

Aunque los procesos térmicos y la presencia de lipasas puede favorecer/iniciar los procesos oxidativos, también las condiciones del envasado pueden ser determinantes. Smet et al. (2009) en estudios llevados a cabo en leche UHT, donde un grupo de muestras tuvo un mayor contenido en insaturados por la alimentación de la vacas y envasada en botella de vidrio o poliuretano, expuestas a la luz y a temperatura ambiente durante 85 días, encontraron que únicamente en el envasado que permitía el paso de O_2 (poliuretano), se produjeron alteraciones atribuibles a procesos de oxidación, mientras que en el resto de muestras no se observaron cambios. A los 60 días de conservación no se detectaron tocoferoles, mientras que la concentración de compuestos prooxidantes procedentes de la riboflavina aumentaron, al igual que los indicadores de compuestos de oxidación primarios a partir de los 14 días (hidroperóxidos) y también secundarios (hexanal procedente de la degradación de ácido linoleico), de forma acusada en las muestras de mayor contenido en PUFAs. También se constató oxidación de proteínas por la disminución de grupos sulfidrilo y aumento de carbonilo.

En conjunto, puede resumirse que existen evidencias de qué tanto los tratamientos térmicos, por conducir el aumento de las condiciones oxidantes de la leche y derivados, como la calidad microbiológica de la materia prima, puesto que existen lipasas microbianas resistentes al procesado térmico, pueden conducir/iniciar a la oxidación lipídica, sobre todo en el caso de ácidos grasos poliinsaturados, y extendiéndose durante el periodo de conservación. Sin embargo en este último punto, la presencia de iniciadores radicálicos como luz, O_2 y metales son un factor a tener en cuenta, especialmente en el caso de los dos primeros factores donde un envasado adecuado puede retrasar o evitar el desarrollo de alteraciones de la fracción lipídica.

OBJETIVOS Y PLAN DE TRABAJO

2. OBJETIVOS Y PLAN DE TRABAJO

Cómo se ha expuesto en la introducción, durante la últimas décadas la investigación nutricional y en particular los alimentos funcionales, han experimentado un importante avance y, en consecuencia, también la evidencia científica sobre la relación dieta-salud. Enfermedades como la obesidad, cardiovasculares, síndrome metabólico, hipercolesterolemia, diabetes etc., relacionadas con un elevado consumo de ácidos grasos saturados y *trans* en países industrializados, están alcanzando actualmente el grado de pandemia. La evidencia científica sobre la importancia de la dieta en la prevención y desarrollo de dichas enfermedades ha proporcionando a los expertos el soporte científico para establecer recomendaciones dirigidas a conseguir una alimentación más saludable.

La grasa láctea se caracteriza por ser mayoritariamente saturada, lo que incide en considerar a la leche y los productos lácteos dentro del grupo de alimentos no saludables. Sin embargo, con las herramientas de investigación disponibles, en las últimas décadas se ha documentado la presencia en grasa láctea de distintos compuestos lipídicos que ejercen una importante actividad biológica. Entre los componentes lipídicos y ácidos grasos bioactivos presentes, cabe destacar el ácido butírico, el ácido linoleico conjugado (CLA), constituyentes de la membrana del glóbulo de grasa como esfingolípidos, vitaminas liposolubles, carotenoides, etc. Estos compuestos ofrecen una potencial aplicación comercial en el desarrollo de alimentos funcionales, encaminados a la promoción de la salud humana y/o en la prevención de enfermedades.

En rumiantes, los ácidos grasos insaturados y poliinsaturados aportados por la alimentación son biohidrogenados por enzimas bacterianas presentes en el rumen; en la glándula mamaría tiene lugar la síntesis del 70% CLA presente en la leche. El conocimiento de estas reacciones posibilita la modificación de la composición de la grasa láctea, potenciando la concentración de aquellos ácidos grasos de interés biológico.

La producción de leche procede en su mayor parte del ganado bovino, sin embargo, otros rumiantes (cabras y ovejas) presentan ciertos aspectos interesantes en la composición de la grasa de su leche, como una mayor concentración de CLA y ácidos grasos de cadena corta.

Por ello, el profundizar en el conocimiento de cómo mejorar el perfil en ácidos grasos de leche y los efectos derivados de la manipulación de la dieta de los rumiantes sigue siendo un área en creciente expansión.

Por otra parte, nuevas vías para la mejora nutricional del perfil de ácidos grasos de la leche, más allá de la sustitución tecnológica, están siendo investigadas, como es el empleo de bacterias lácticas capaces de transformar substratos lipídicos en ácidos grasos poliinsaturados de reconocido efecto biológico como el CLA.

No obstante, en el caso de alimentos funcionales, enriquecidos, suplementados y/o fortificados en su fracción lipídica, debe existir el compromiso de garantizar la estabilidad de estos compuestos durante los procesos tecnológicos y de conservación durante su vida útil. Las reacciones que producen su alteración, tienen como sustrato principal a los ácidos grasos insaturados, de carácter oxidativo, generando radicales y compuestos que tienen un efecto nocivo en la salud al alcanzar determinadas concentraciones. Otras reacciones producen la isomerización de los ácidos grasos en sus formas *trans* durante el procesado térmico a alta temperatura. En los fosfolípidos presentes en grasa láctea, las alteraciones aparecen tras la disrupción de la membrana del glóbulo graso por efecto de la homogenización o el tratamiento térmico de la leche, posibilitando su hidrólisis o su interacción con las proteínas.

En la bibliografía, son escasos los estudios que determinan alteraciones o modificaciones en el contenido en ácidos grasos u otros compuestos lipídicos en leche y productos lácteos, cuya fracción lipídica ha sido mejorada de forma natural o fortificada y que podrían atribuirse a reacciones iniciadas durante la elaboración, tratamiento o conservación.

Así, ante la tendencia a aumentar el contenido en ácidos grasos insaturados con reconocida actividad biológica en lácteos y derivados, y las evidencias, aunque insuficientemente investigadas, de que tanto el procesado industrial

como la conservación durante el periodo de vida útil del producto, pudieran tener lugar reacciones que condujeran a la alteración de su fracción lipídica (ácidos grasos y fosfolípidos) se plantea en esta Tesis Doctoral el siguiente OBJETIVO GENERAL:

Mejora nutricional del perfil lipídico de leche y productos lácteos así como la evaluación de las posibles alteraciones en su contenido o distribución provocadas durante su procesado convencional (térmico) o no convencional, así como durante su posterior conservación.

Para la consecución de dicho objetivo, se plantean los siguientes objetivos parciales:

1. Desarrollo, optimización y validación de métodos cromatográficos para el estudio de la fracción lipídica de productos lácteos.
2. Estudio de la mejora nutricional del perfil lipídico de la leche mediante la incorporación a la dieta de rumiantes de suplementos ricos en PUFA, o mediante empleo de bacterias lácticas con capacidad para transformar el ácido linoleico en isómeros del ácido linoleico conjugado (CLA).
3. Evaluación del efecto de los tratamientos industriales convencionales (pasterización, UHT y esterilización) frente a los no convencionales (microondas, altas presiones y homogenización a alta presión) sobre el perfil lipídico y el contenido en fosfolípidos de leche de vaca, oveja y cabra.
4. Determinación de la posible alteración de la fracción lipídica de fórmulas infantiles en polvo y derivados lácteos de alto contenido en CLA durante su periodo de conservación.

RESULTADOS Y DISCUSIÓN

3. RESULTADOS Y DISCUSION

Los resultados de esta tesis y su discusión, han sido obtenidos de la realización de diferentes trabajos, presentándose en los siguientes apartados.

3.1. Desarrollo, optimización y validación de métodos cromatográficos para el estudio de la fracción lipídica de productos lácteos.

- **Rodríguez-Alcalá, L.M**, María V. Calvo and Javier Fontecha. Analysis of milk fat, vegetable and fish oil fatty acids using a short time GLC method. Manuscrito en preparación.

- **Rodríguez-Alcalá, L.M** and Fontecha, J. Major lipid classes separation of buttermilk, and cows, goats and ewes milk by HPLC-ELSD focused on the phospholipid fraction. Enviado: Journal of Chromatography A.

3.2. Estudio de la mejora nutricional del perfil lipídico de la leche mediante la incorporación a la dieta de rumiantes de suplementos ricos en PUFA, o mediante empleo de bacterias lácticas con capacidad para transformar el ácido linoleico en isómeros del ácido linoleico conjugado (CLA).

- María V. Calvo, **Rodriguez-Alcalá, L.M.**, Juliana Kives, Jesús Romero, and Javier Fontecha. Influence of feeding linseed at different levels on fatty acid profile focused on the CLA isomers composition of goat milk. Enviado: European Food research and technology.

- **Rodríguez-Alcalá,** L.M, Teresa Braga, Ana Gomes, F. Xavier Malcata and Javier Fontecha. Quantitative and qualitative determination of CLA produced by Bifidobacterium and LAB combining spectrophotometric and Ag+-HPLC techniques. Enviado: Food Chemistry.

3.3. Evaluación del efecto de los tratamientos industriales convencionales (pasterización, UHT y esterilización) frente a los no convencionales (microondas, altas presiones y homogenización a alta presión) sobre el perfil lipídico y el contenido en fosfolípidos de leche de vaca, oveja y cabra.

- **Rodríguez-Alcalá, L. M.**, L. Alonso and J. Fontecha. Influence of heat treatments, high pressure and microwave processing of naturally PUFA enriched milk on CLA isomers distribution and trans fatty acids content. Manuscrito en preparación.

- **Rodríguez-Alcalá, L. M.**, Harte, F. & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. Innovative Food Science and Emerging Technologies 10, 32-36.

- **Rodríguez-Alcalá, L.M** and Fontecha, J. Cow milk processed at very high pressure: effects on the fatty acids and phospholipids composition. Manuscrito en preparación.

3.4. Determinación de la posible alteración de la fracción lipídica en fórmulas infantiles en polvo y derivados lácteos de alto contenido en CLA durante su periodo de conservación.

- **Rodriguez-Alcala, L. M.** & Fontecha, J. (2007). Hot Topic: Fatty Acid and Conjugated Linoleic Acid (CLA) Isomer Composition of Commercial CLA-Fortified Dairy Products: Evaluation After Processing and Storage. Journal of Dairy Science 90, 2083-2090.

- **Rodriguez-Alcala, L. M.**, Garcia-Martinez, M. C., Cachon, F., Marmesat, S., Alonso, L., Marquez-Ruiz, G. & Fontecha, J. (2007). Changes in the Lipid Composition of Powdered Infant Formulas during Long-Term Storage. Journal of Agricultural and Food Chemistry 55, 6533-6538.

3.1. Desarrollo, optimización y validación de métodos cromatográficos para el estudio de la fracción lipídica de productos lácteos.

Analysis of milk fat, vegetable and fish oil fatty acids using a short time GLC method

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Abstract:

Gas chromatography is the main analytic technique for the fatty acid analysis, involving several steps as sample isolation, preparation, generally derivatization, and injection. Using theses methods most of the time is used in the chromatographic run, this point has been improved with the development of new columns of lower internal diameter and optimization of the temperature programs resulting in short chromatographic runs, very useful in the routine analysis. In the current work the VF23ms column suitable for fast analysis is tested versus the wide-used CPSil 88; sunflower, safflower, soya, tonalin®, tung and fish oils as well as milkfat were assayed using two thermal programs, one suitable for samples with complex composition in trans fatty acids as milk fats. The results showed that tested conditions are suitable for the fatty acids analysis.

1 Introduction

Current nutritional patterns are characterised by a high animal fat and seed oil intake, resulting towards diets of high contents in saturated and *trans* fatty acids.

Several researches have demonstrated its link among the development of certain diseases as cardiovascular disorders, metabolic syndromes [1, 2] and even some kinds of cancer [3], all this is becoming in a serious health problem.

In the other hand, other investigations have concluded that certain fatty acids and lipid compounds are far of playing a harmful role. Even more they can be tools to improve or keep consumer's health [4, 5]. Examples of all this are the anticarcinogenics activities of butyric [6], rumenic [7] and eleostearic acid [8], antiatherogenic of Oleic [9] and the omega 3 [10] family and for the latter one, their important contribution to the development of infant nervous system [11] as well as its relation in certain mental diseases associated to lower intakes [12].

The quantitative and qualitative determination of fatty acids is mainly carried out by applying gas chromatography techniques with previous derivatization of the sample to fatty acid methyl sters (FAMEs). In those conditions, the capillary columns used have polar stationary phases and lengths from 30 to 100 meters. In complex samples as milk fat, comprising fatty acids from 4 to 24 atom carbon chains, the optimum separation of the chromatographic peaks, led to elevated run times. Furthermore, *trans* fatty required of preparative techniques as TLC or long gas chromatography runs as well as CLA isomers, completely resolved by Ag^{+}-HPLC but not using GLC.

Currently are available new capillary columns with lower particle size diameters allowing considerable reduction of the time of analysis. However most of the methodologies

fastest analysis reported (1-4 total runtime) are based in the use of hydrogen as carrier gas and slightly modified equipments [13, 14].

In the present study are presented two temperature programs (one suitable for dairy fats and the separation of trans fatty acids and the other for less complex samples as vegetable oils) using VF23ms capillary column (30 m x 0.25 mm i.d.x 0.25 μm film thickness) and Helium as carrier gas. The temperature programs were tested using milkfat and safflower, sunflower, soja, tung, tonalin® and fish oils and compared with those results obtaining using a long capillary column CPSil-88 (100 m × 0.25 mm i.d. × 0.2 μm film thickness) in order to be used as routine methods. The latter column is one of the most employed in the fatty acids analysis in order to its resolution capability.

The aim of the present work was carried out a comparative analysis of working conditions, using two capillary columns, in order to obtain a routine methodology that reducing the run times had a minimal lost in the chromatographic resolution of the peaks.

2 Materials and Methods

2.1 Chemicals

All the reagents used in these experiments were analytical grade grade: Hexane and Methanol (LabScan, Dublin, Ireland), Potassium hydroxide and Sodium sulphate-1 hydrate (Panreac, Barcelona, Spain), reference milk fat butter BCR-164 (EU Commissions; Brussels, Belgium, purchased to Fedelco Inc., Madrid, Spain) and glyceryl tritridecanoate (internal standard in milk sample) and triheptadecanoate (internal standard in the oil samples) (Sigma, St. Louis, MO).

2.2 Samples

To test the feasibility of the experimented methods using a VF23 ms capillary column, a wide range of samples was selected; Sunflower, safflower, butter and Soya oils were purchased at a local market, Tung oil from Sigma Aldrich (St. Louis, MO), Fish oil kindly provided by… as well as tonalin® by Cognis (Illertissen, Germany).

2.3 Preparation of FAMEs

FAMEs were prepared by base-catalyzed methanolysis of the lipids using 2N KOH in methanol as described by International Standard method ISO-IDF [15].

2.4 GC-FID analysis.

2.4.1 Conventional Analysis

FAMEs were analyzed on an Agilent chromatograph (model 6890N, Palo Alto, CA, USA) fitted with a flame ionization detector (FID). Fatty acids were separated using CP-

Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, The Netherlands). The column was held at 100°C for 1 min after injection, temperature-programmed at 7°C/min to 170°C, held there for 55 min, and then temperature-programmed at 10°C/min to 230°C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 214 kPa (30 Psig) and a split ratio of 1:20. The injector temperature was set at 250ºC and the detector temperature was set at 270ºC. Injection volume was 0.5 μL. To obtain response factors (Rf), reference butter fat BCR-164 was injected. For quantitative purposes tritridecanoin was added both in test samples and reference butter fat.

2.4.2 Quick analysis

Two time-reduced GC analysis on a VF-23, fused-silica capillary column (30 m x 0.25 mm i.d.x 0.25 μm film thickness, Varian, Middelburg, Netherlands), was developed. The FAME were analysed on a Clarus® 500 chromatograph (Perkin Elmer, Beaconsfield, UK).

Temperature program 0 (P0): The column was held at 160ºC, 14 min after injection, temperature-programmed at 45ºC/min to 210ºC, held for 5 min. Helium was the carrier gas with a column inlet pressure set at 120.5 kPa (15 psig) and a split ratio of 1:50. The injection volume was 0.5 μl. Total run time was of 25 min.

Temperature program 1 (P1): The column was held at 120ºC for 1 min after injection, temperature-programmed at 10ºC/min to 140ºC, then temperature-programmed at 15ºC/min to 180ºC and last ramp at 5ºC to 240ºC held there for 3 min. Helium was the carrier gas with a column inlet pressure set at 120.5 kPa (15 psig) and a split ratio of 1:50. The injection volume was 0.5 μl. Total run time was of 17 min.

2.5 Validation parameters

Intra and inter-assay precision was assessed by the coefficient of variation (CV) relative to 3 replicates per sample and carried out by the same operator and results were accepted if they were under 10% [16].

3 Results and discussion

3.1 Comparison of the qualitative results

Gas chromatography is the main technique used in qualitative and quantitative analysis of fatty acids present in fats or edible oils. But such tests require prior stages of sample preparation in which fatty are derivatized to more volatile compounds such as methyl esters (FAMEs). Given the complexity of the milk fat, for the analysis of FAMEs have been used capillary columns with high selectivity polar phases (such as cyano-propyl) and length (50 to 130 m) to obtain the best possible resolution between the different components present (eg differentiation between cis and trans).

In the labor where this work has been carried out, methodologies were available for GC-FID analysis by using CPSil-88 column of 100 meters long [17, 18]. which one can obtain a complete description of fatty acid composition of milk fat, given its high resolution capacity. However, analysis times used for each sample are also very long, in the particular case of this column reaches 105 minutes, so that to carry out a routine analysis of many samples it was needed the optimization of the method but without influence in the resolution. To this end, the column VF23ms was used with the same phase but 30m x 0.25mm x 0.25µm (Varian) and two temperature programs. One of them (P0) was developed for the analysis of milk fat in which the aim is a good elution and

147 resolution of short-chain fatty acids, trans compounds and conjugated linoleic CLA. For
148 the optimization procedure was used a reference milk fat CRM164 (Fedelco, Madrid).
149 Also the procedure was validated for studying the composition of various vegetable and
150 fish oils by optimization of temperature program used (P1).
151 The elution orders was known by comparing the injection of a reference butterfat sample
152 (BCR-164) in both the GC installed with the CPSil-88 column and the one with the
153 VF23ms, using the temperature programs described in the methods and materials section.
154 The fatty acid composition of the milkfat where compounds of short, medium and long
155 chain are present, made possible to accurate the temperature programs to be used using
156 the VF23ms column.
157 The analysis of the reference CRM164 fat showed that CPSil-88 column (Figure 1A)
158 achieves the separation of fatty acids C18:1 trans in 5 distinct peaks (C18:1 t6-t8, C18:1
159 t9, C18:1 t10, C18:1 t11, C18:1 t12-14t) being able to observe the trace presence of C18:
160 1 t4-C18: 1 t5. It is also possible to differentiate minor cis oleic acid (C18:1 cis 9)
161 compound as C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14 (which coelutes with t16) and
162 the C18:1 c15. The Minor isomers of linoleic acid (C18:2 c9, c12) can be detected as
163 C18:2 t,t NMID (non methylene interrupted double bonds), C18:2 t9,t12, C18:2 c9,t12 ,
164 C18:2 t11,c15, C18:2 t9,c12 and C18:2 t9,c15. As for the CLA isomers, in addition to the
165 presence of rumenic acid (C18: 2 c9, t11) in this column and working conditions it is
166 possible the quantification of C18:2 t8,10c, C18:2 t11,13c, C18:2 t10,12c, C18:2 9c,11c,
167 C18:2 10c,12c, C18:2 11c,13c and C18:2 *trans*, *trans* (total). It also detected CLnA the
168 isomers (one of them identified as C18: 3 c9, t11, t13). In the analysis of FAMEs from
169 vegetable and fish oils through the application of this column, it can also be determined,
170 in addition to the acids of medium chain length, a very large number of long and very
171 long chain length fatty acids as EPA , DPA and DHA, with the same program previously

172 used for milk fat (Table 2). The use of the VF23ms column with the temperature program
173 P0 to CRM164 analysis reveals that the oleic acid trans compounds are resolved in three
174 peaks C18:1 t4-t9, C18:1 t10 and C18:1 t11 (TVA) , while compounds C18:1 cis appear
175 in the order C18:1 c11, C18:1 c12 and C18:1 c13 + c14. The elution of compounds of
176 C18:2 was trans,trans; cis,trans/trans,cis and cis,cis. Using temperature program P1 for
177 the analysis of vegetable and fish oils was observed that it is possible to differentiate the
178 isomers C18: 2 t,t NMID, C18:2 t9,t12, C18:2 c9,t13, C18:2 c9,t12 and C18:2 t11, 15c,
179 since in these conditions all these fatty acids had different retention times. Under these
180 conditions it was also possible to differentiate the isomers of CLA RA, C18:2 t10,c12,
181 C18:2 c,c (total) and C18:2 t,t (total). Also isomers of CLnA was determined in addition
182 to AA, EPA, DHA, DPA.
183 Several previous studies highlight the difficulty of reducing analysis time without
184 compromising the resolution of various fatty acids. In works carried out with columns of
185 10 m, with hydrogen as carrier gas, the analysis of milk fat can be obtained within 4
186 minutes. However, the C18:1 trans and cis are not separate and co-eluting in one peak,
187 while from the CLA isomers, only RA is detected [13]. In other studies using larger
188 columns (40m) and helium as the carrier (26-30 min) show that in the analysis of
189 standards and milk fat from human milk, the acid C18:1 trans and cis, C18:2 trans trans
190 and C18:2 cis,trans-trans,cis eluted in one peak each, not being able to obtain a more
191 detailed composition, whereas for the compounds of CLA is possible to quantify RA,
192 C18:2 c11,t13 ,C18:2 t10,c12 and C18:2 t,t CLA [19, 20]. Other fast chromatography
193 studies with columns of 30 meters, show that in the analysis of mixtures of
194 chromatographic standards, the isomers of C18:1 cis and trans fatty acids overlap and can
195 not be distinguisehd and that for the C18:2 although separable trans,trans from cis,cis, the

196 cis,trans and trans,cis coelutes, being the situation similar for the compounds of CLA
197 [21].
198
199 **3.2 Comparison of the quantitative results**
200
201 • **Intra-assay results:**
202
203 All analysed samples had the expected fatty acids compositions (tables 1 and 2); milk fats
204 are characterised by the presence of saturated fatty acids (myristic, palmitic and estearic)
205 and high oleic content [22]. In safflower, sunflower and soja oils, linoleic acid was the
206 major compound, with oleic in MUFA fraction and palmitic in SFA [23]; tonalin® oil
207 was mainly composed by conjugated linoleic acid isomers [17] as tung oil of conjugated
208 linolenic acid [24]. In fish oils the main fatty acids were palmitic, oleic and linoleic and
209 the presence of omega compounds as stearidonic (C18:4), EPA and DHA fatty acids [25,
210 26].
211 The results show compositions and contents similar to others previously reported for milk
212 fat [22], sunflower, safflower and soybean [23], Tonalin ® [17], tung [24] and fish [25].
213 As showed in the enclosed results, the fatty contents obtained applying program 0 to
214 milkfat samples and program 1 to vegetable oils are acceptable in order to the coefficient
215 of variation (CV) values of the intra assay analysis are below the 10 % allowance limit
216 proposed by Horwitz [16] and showing the reproducibility of the results obtained
217 applying the assayed methods. Furthermore, as significant benefit, indicating that the
218 time required for the elution of all peaks present in milk fat, vegetable oils and fish was
219 less than 17 min.
220

- **Inter-assay results:**

As mentioned above, the aim of the present work was to develop a GC method using the VF23ms column to reduce the time of analysis. Then is compulsory to compare the results obtained using the latter column with those of a routine and well established method (CPSil-88 capillary column). For this purpose the coefficient of variation with the data from both columns was calculated.

Short chain fatty acids (SCFA) are characteristic compounds of the milk fat. As they are extremely volatile in the form of FAME, their analysis is a critical issue in this kind of samples. The proposed temperature program was able to detect those compounds and their contents when comparing VF23ms with CPSil-88 columns (CV below 1%; table 1).

In overall, the results are close to those obtained when using CPSil-88 instead of the smaller resolution of the VF23ms and the need to accomplish bigger dilutions of the samples. Myristic, palmitic and stearic acids are the major saturated moieties in milkfat and as with SCFA, the obtained CV were under 1% (0.1, 0.5 and 0.6 respectively). For the TFA the RSD values were 8.3 (C18:1 *trans* 6- *trans* 9), 1.9, (C18:1 *trans* 10) and 7.4 (C18:1 *trans* 11) although bigger than in other fatty acids are below the allowance variation limit (10%). Oleic and linoleic acid had similar results (5.8%, 4.7% respectively). CLA compounds, interesting by their biological activities, can be detected and quantified in the assayed conditions with a variation of the total content regarding to CPSil-88 of 4.9%. The results are suitable to use the temperature program called 0 in the routine analysis of milkfat.

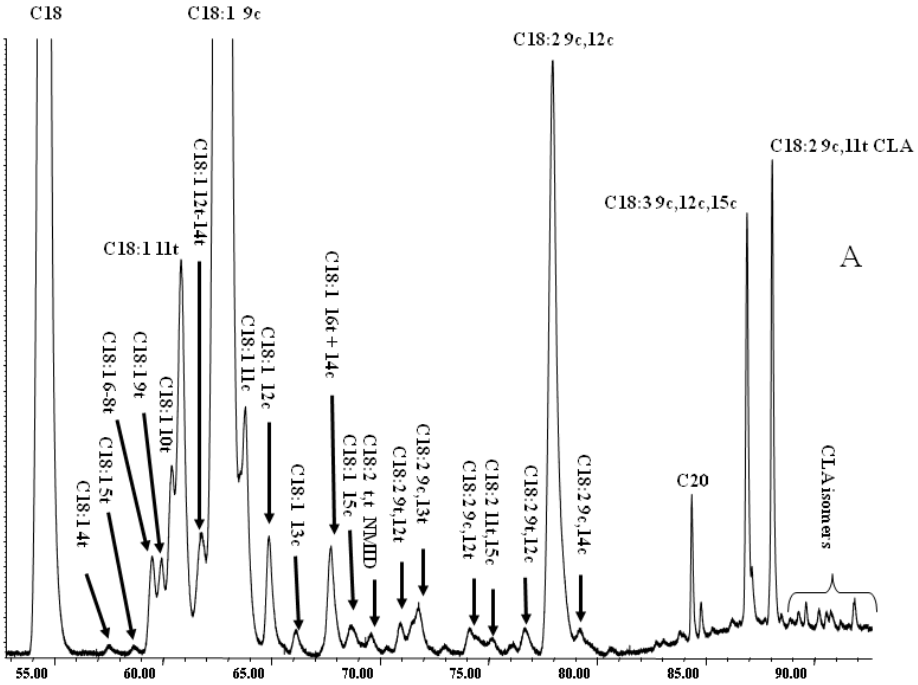
The program 1 (P1) was tested in vegetable and fish oils and also compared with the results of CPSil-88 with CV as parameter test. Here the results fitted well with those from

246 the CPSil-88 as presented forward: In safflower oil samples, the CV values for the major
247 compounds were 5% (palmitic acid), 9.9% (stearic acid) and 2.7% (oleic and linoleic
248 acid). Sunflower composition had the same major fatty acids than the latter vegetable oil
249 and the variations in the inter assay test also were corrects: palmitic with 4.4%, stearic
250 with 9.1%, 2.2 for oleic and 3.5 for linoleic.
251 In the soja oil samples (Table 4), the relative standard deviations of the main fatty acids
252 palmitic (2.9%), stearic (3%), oleic (0.7%), linoleic (0.9%) and linolenic (6.6%) assessed
253 using the shorter column correlated well with the reference contents.
254 Tung oil is obtained from a seed of a chinese tree and is characterised by the presence of
255 conjugated linolenic fatty acids (CLnA). As in the other vegetable oils described, the
256 method showed contents alike to those in the reference column. When calculating the
257 relative standard deviations for the CLnA isomers, the values were 1.55 and 4.1%
258 respectively for each isomer detected.
259 Tonalin® oil is obtained synthetically from safflower through the alkali isomerization of
260 linoleic acid. The CV for the major compounds was, 4.2% for stearic, 3.5% for oleic acid.
261 In the main CLA isomer the inter assay test have as CV values of 1.7 and 4.3
262 respectively. The other two compounds obtained in using the VF23ms column had values
263 of 0.3% and 4%.
264 Fish oil is also an interesting lipid source due to the presence of some polyunsaturated
265 fatty acids as stearidonic, arachidonic, EPA, DPA and DHA fatty acids belonging to the
266 n3 and n6 families. Applying program 1 to this samples and comparing with those of the
267 reference method, the contents in both situation correlated well as they were below a 10%
268 of variation: C18:4, 2.5%; C20:4, 9.3%; C20:5, 0.1%, C22:5, 5.1% and C22:6, 1.6%. For
269 the main compounds myristic, palmitic, stearic, oleic, C18:1 *cis* and linoleic acid, the
270 variations with the CPSil-88 contents were 2.6, 7, 6, 6.8, 8.5 and 0.8% respectively.
271

272

273 **4 Conclusions**

274 With the analytical conditions described in this paper has been perfomed a quick analysis
275 of the composition of FAMES both in milk fat and different edible oils, showing good
276 resolution and reproducibility for minor compounds and isomers of the main components
277 such as oleic, linoleic , linoleic and CLA. It is therefore possible to be used as a routine
278 method for studying the fatty acid profile of a large number of samples.



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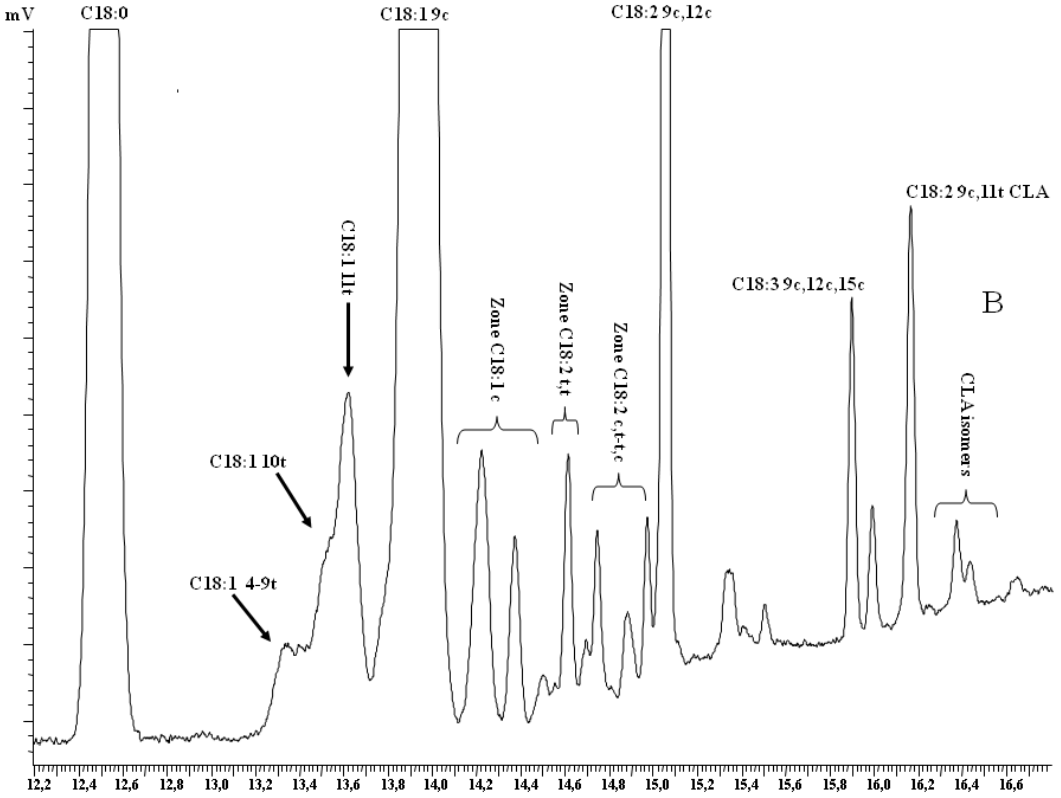

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282 **Figure 1.** Fatty acid profile of the C18:1 and C18:2 region in milkfat : A, CPSil-88 column; B, VF23ms
283 column.
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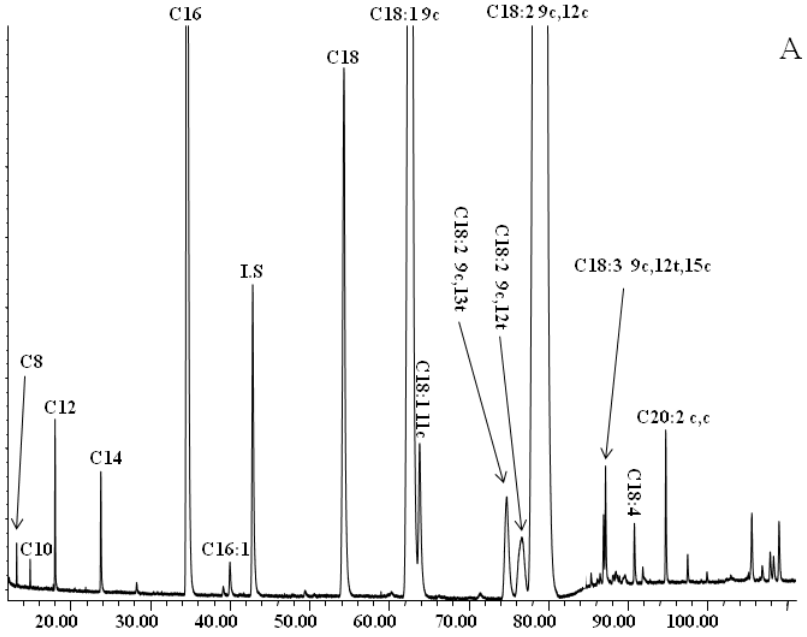


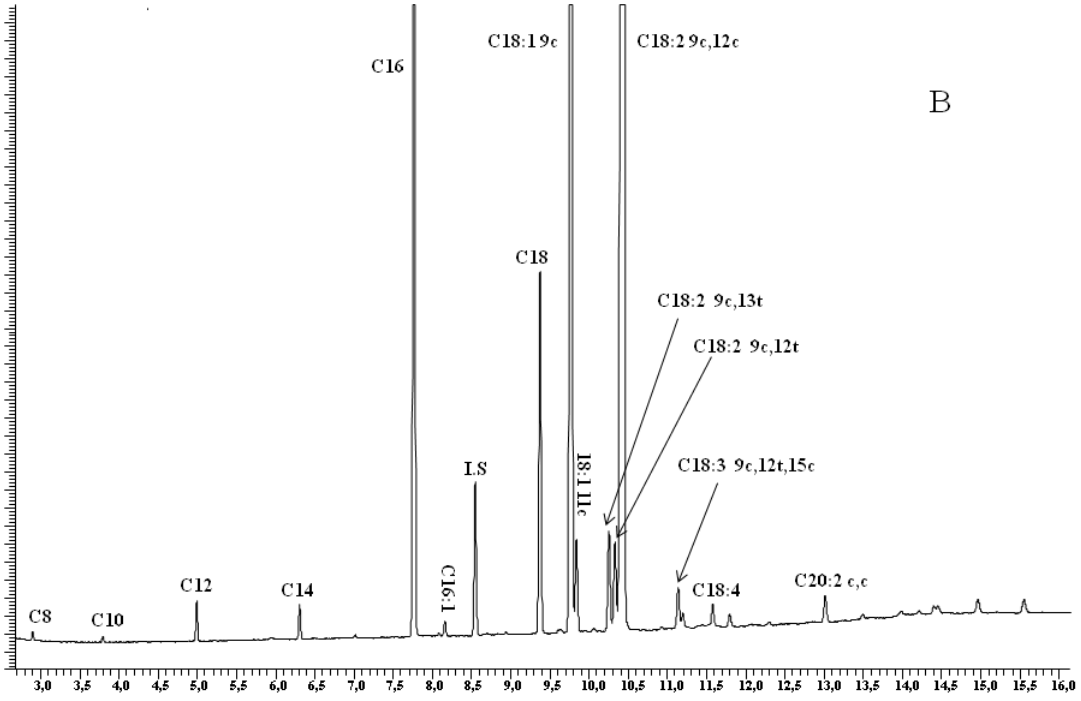
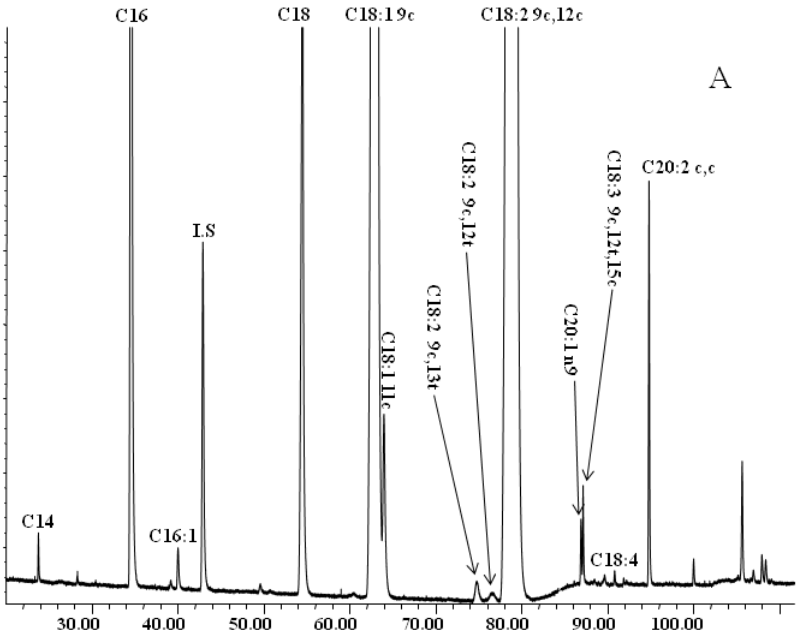


Figure 2. Fatty acid profile in safflower oil: A, CPSil-88 column; B, VF23ms column.



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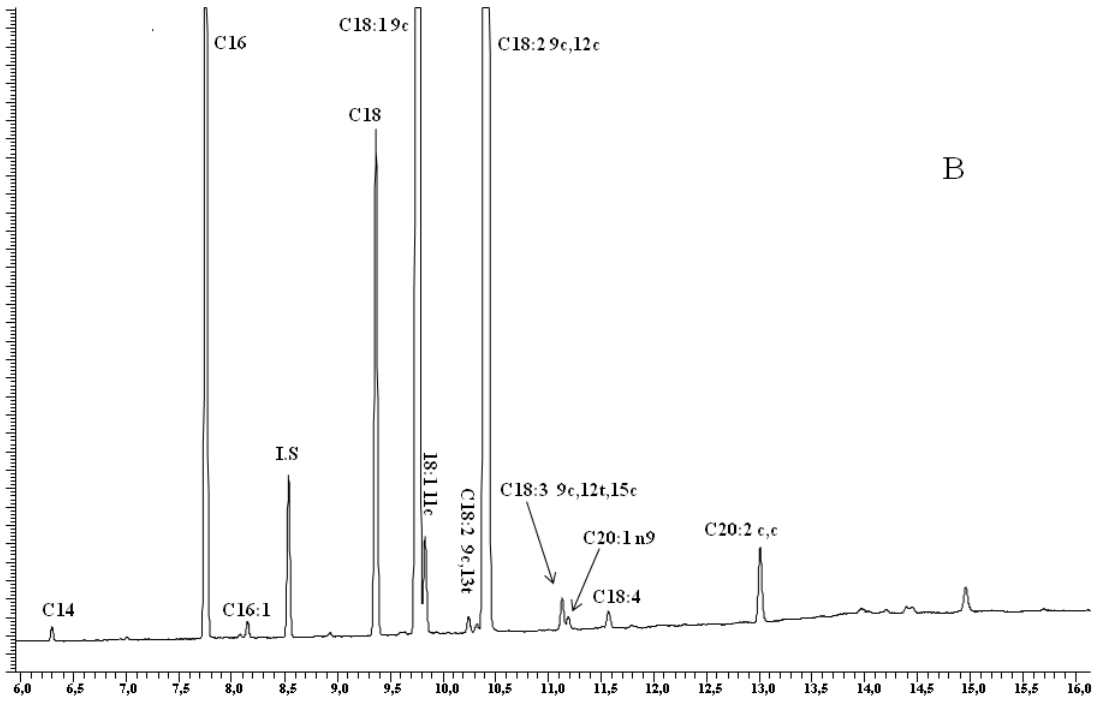

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294 **Figure 3.** Fatty acid profile in sunflower oil: A, CPSil-88 column; B, VF23ms column.
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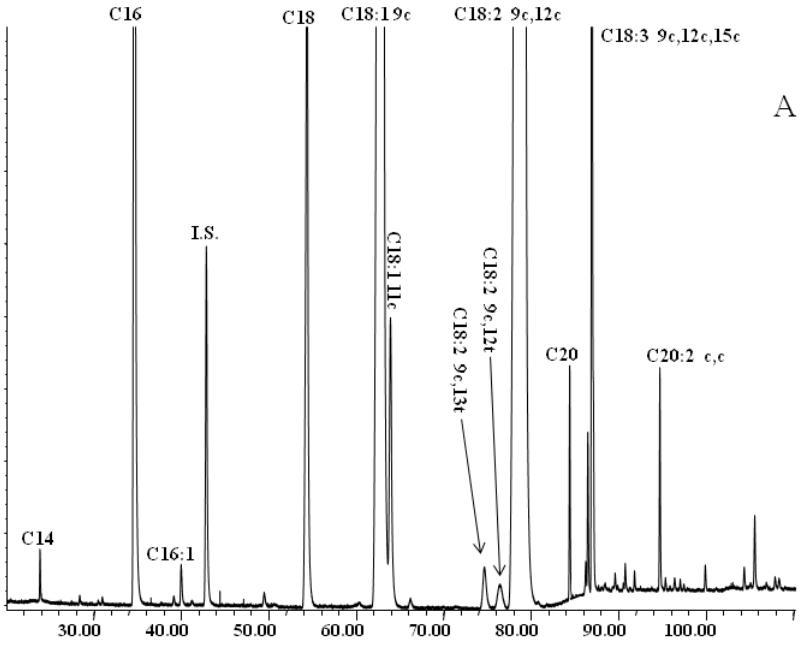

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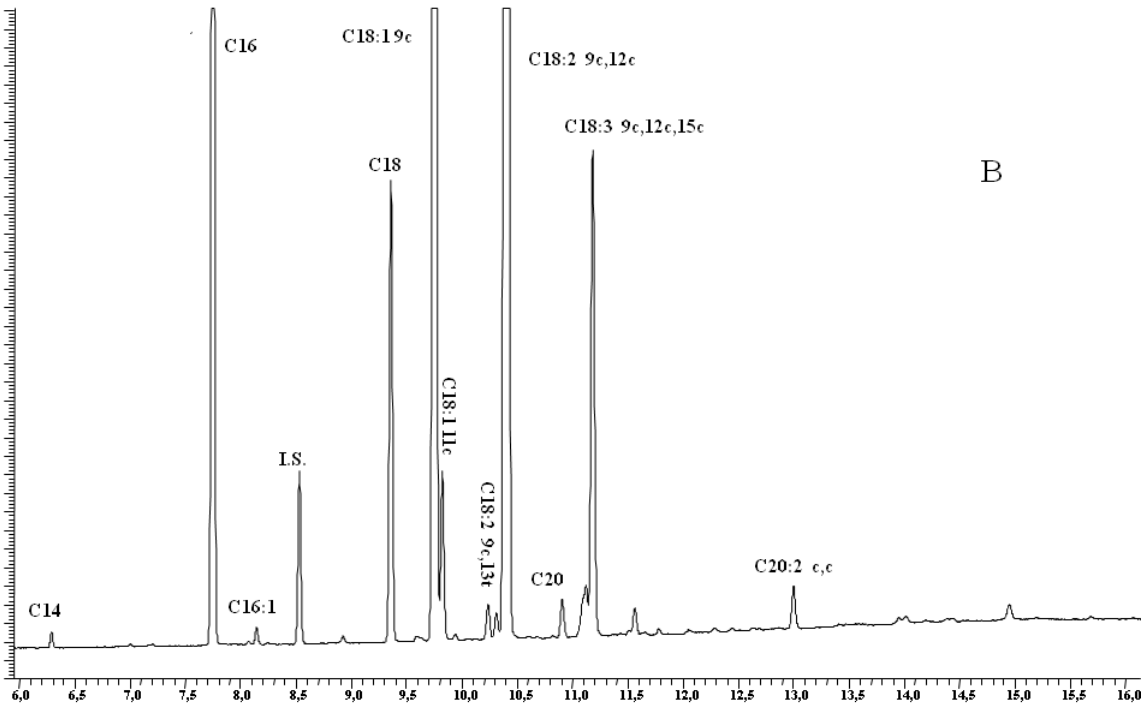

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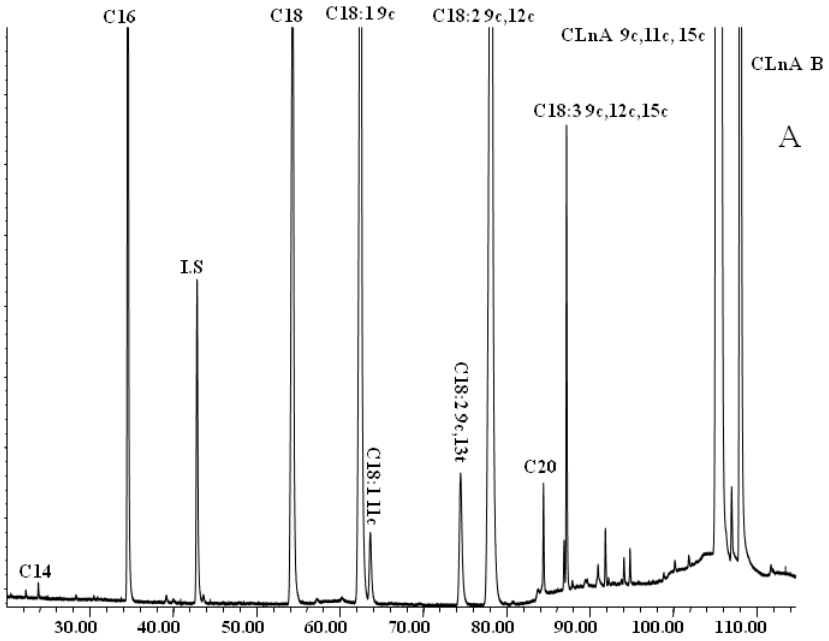
302 **Figure 4.** Fatty acid profile in soya oil: A, CPSil-88 column; B, VF23ms column.



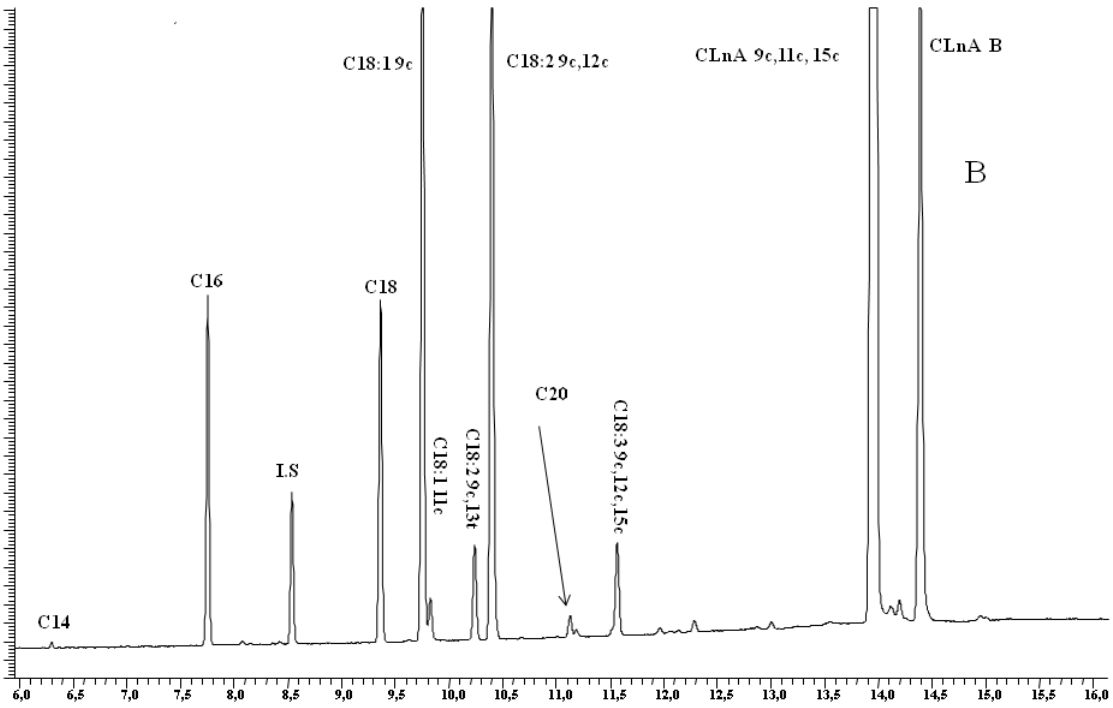
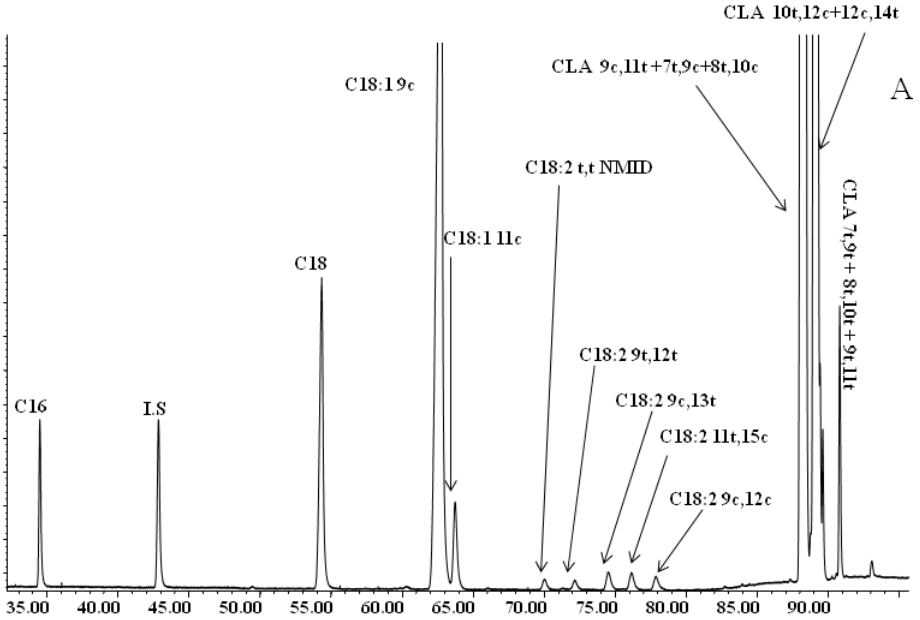


Figure 5. Fatty acid profile in tung oil: A, CPSil-88 column; B, VF23ms column.



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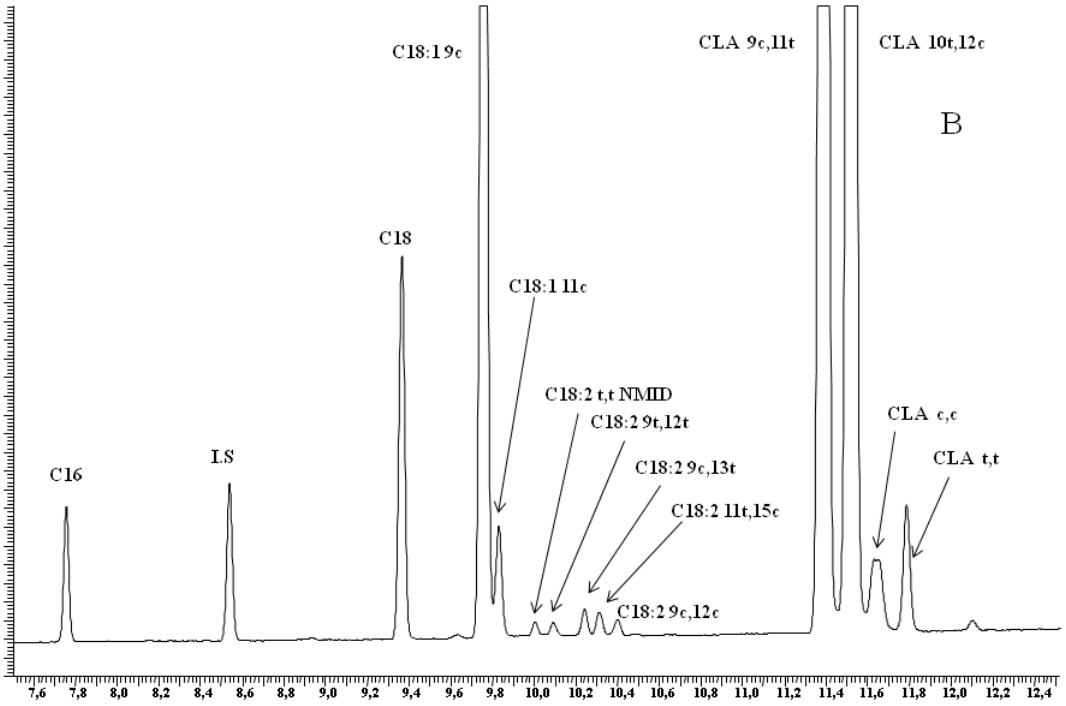

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312 **Figure 6.** Fatty acid profile in tonalin® oil: A, CPSil-88 column; B, VF23ms column.
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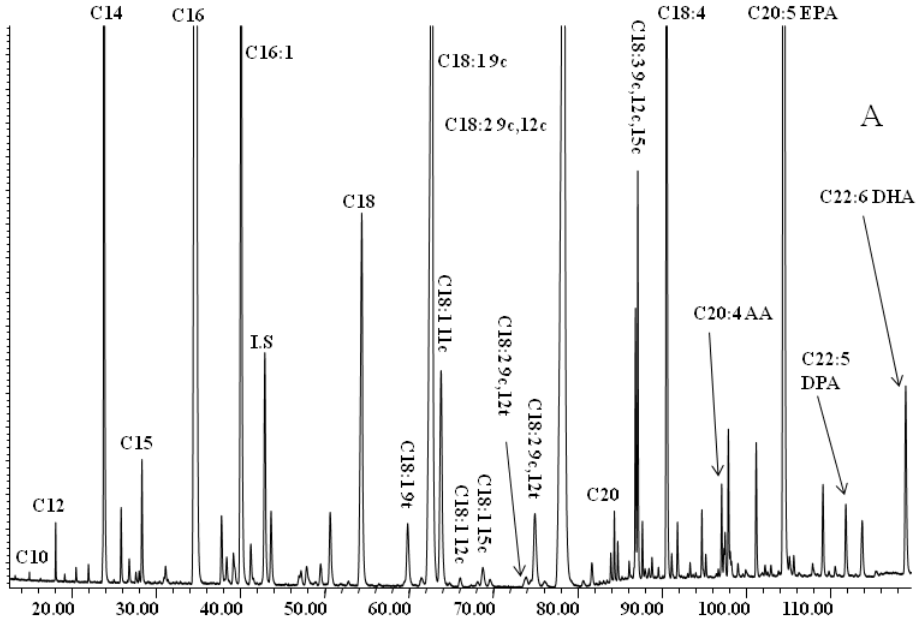

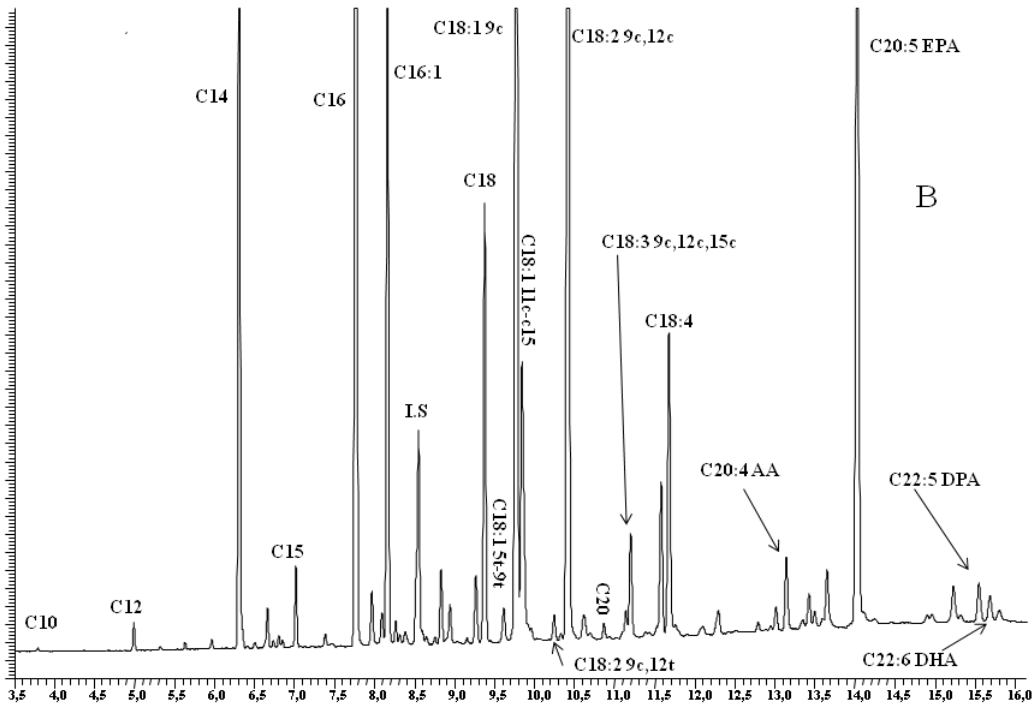

Figure 7. Fatty acid profile in fish oil: A, CPSil-88 column; B, VF23ms column.

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Table 1. Fatty acid composition (g FA/ 100 g F) of milkfat samples

CP-Sil88								VF23ms				
Fatty acids	**Rt (min)**	**Mean**	**CV[1]**	**Fatty acids**	**Rt (min)**	**Mean**	**CV[1]**	**Fatty acids**	**Rt (min)**	**Mean**	**CV[1]**	**CV[2]**
C4	11.70	3.50	2.216	**C18:1 15c**	68.76	0.12	3.920	**C4**	2.16	3.47	2.204	0.744
C6	12.21	2.29	0.691	**C18:2 t,t NMID**	70.96	0.06	4.267	**C6**	2.25	2.32	1.633	0.860
C8	13.14	1.27	0.794	**C18:2 9t,12t**	71.77	0.16	1.797	**C8**	2.44	1.29	0.912	0.562
C10	14.85	2.77	0.846	**C18:2 9c,13t**	74.15	0.09	4.662	**C10**	2.81	2.78	0.561	0.271
C10:1	16.24	0.26	1.611	**C18:2 9c,12t**	75.17	0.04	9.980	**C10:1**	3.01	0.24	1.305	6.215
C12	18.00	3.12	0.572	**C18:2 11t,15c**	76.16	0.02	4.126	**C12**	3.52	3.13	0.357	0.368
C13i	19.09	0.03	7.159	**C18:2 9t,12c**	76.69	0.07	5.047	**C14i**	4.46	0.09	2.823	1.786
C13ai	19.59	0.09	3.311	**C18:2 9c,12c**	77.96	1.99	0.138	**C14**	4.89	10.27	0.368	0.135
C14i	21.95	0.09	2.603	**C18:2 9c,15c**	79.23	0.08	7.296	**C15i**	5.40	0.25	4.859	0.593
C14	23.86	10.25	0.379	**C20**	84.34	0.09	0.614	**C14:1**	5.54	0.91	0.727	0.182
C15i	25.84	0.26	0.410	**C18:3 n6**	84.76	0.02	2.793	**C15ai**	5.63	0.47	0.907	2.271
C14.1t	26.28	0.02	12.838	**C20:1 t**	86.20	0.02	10.211	**C15**	6.00	0.99	0.360	1.251
C15ai	26.81	0.48	0.963	**C18:3 9c,12c,15c**	86.88	0.36	9.081	**C16i**	6.70	0.19	5.532	3.958
C14:1	27.61	0.91	0.551	**C20:1 n9**	87.10	0.06	8.134	**C16**	7.53	31.08	0.201	0.543
C15	28.33	0.98	0.515	**C18:2 c9,11t+7t,9c+8t,10c**	88.02	0.44	3.734	**C17i**	8.28	0.20	4.949	44.744
C16i	31.14	0.18	2.337	**C21**	88.45	0.02	8.930	**C16:1**	8.47	1.55	1.127	2.099
C16	34.88	30.85	0.145	**CLA 11c,13t**	89.26	0.02	7.886	**C17ai**	8.94	0.55	2.804	35.279
C17i	38.35	0.39	1.471	**CLA 10t,12c+12c,14t**	89.60	0.03	10.498	**C18**	12.54	10.74	0.228	0.634
C17ai	39.15	0.33	0.821	**CLA 7c,9c+8c,10c+9c,11c**	90.18	0.02	10.063	**C18:1 4t-9t**	13.34	0.36	4.488	8.317
C16:1	40.04	1.60	0.161	**CLA 10,12c**	90.55	0.02	10.540	**C18:1 10t**	13.54	0.45	5.805	1.884
C17	42.84	0.57	1.197	**CLA 11c13c+12c,14c**	90.76	0.03	10.781	**C18:1 11t**	13.63	1.19	4.946	7.396
C17:1c	49.50	0.29	1.725	**CLA 11t,13t**	91.22	0.01	10.210	**C18:1 9c**	13.97	20.68	0.129	5.834
C18	54.68	10.64	0.124	**CLA 7t,9t+8t,10t+9t,11t**	91.86	0.04	10.839	**C18:1 11c**	14.23	0.94	1.427	2.122
C18:1 5t	57.48	0.04	5.214	**C20:2 c,c**	93.11	0.03	2.692	**C18:1 12c**	14.38	0.33	1.824	8.108
C18:1 4t	58.63	0.02	9.154	**C22**	94.70	0.05	4.154	**C18:1 13c+14c**	14.62	0.31	1.598	9.079
C18:1 6t-8t	59.50	0.16	2.167	**C20:3 n6**	95.20	0.10	4.010	**c18:2 t,t**	14.75	0.15	5.589	7.672
C18:1 9t	59.91	0.24	3.324	**C20:4**	97.89	0.16	4.977	**c18:2 c,t**	14.89	0.14	7.930	3.904
C18:1 10t	60.39	0.46	1.897	**C20:3 n9**	101.22	0.02	7.947	**c18:2 t,c**	14.98	0.10	4.901	9.469
C18:1 11t	60.83	1.32	1.028	**C20:5 EPA**	104.37	0.04	6.788	**C18:2 9c,12c**	15.06	2.12	0.279	4.716
C18:1 12t-14t	61.76	0.43	0.924	**C22:5 DPA**	105.55	0.03	3.973	**C18:3 9c,12c,15c**	15.90	0.41	2.840	8.291
C18:1 9c	62.98	19.04	0.194	**C22:6 DHA**	118.91	0.05	3.200	**CLA c9,11t**	16.17	0.45	1.909	4.897
C18:1 11c	63.79	0.91	1.125	**SFA**		68.24	0.284	**CLA other**	16.38	0.14	5.233	1.256
C18:1 12c	64.89	0.37	1.046	**MUFA**		26.74	0.195	**C20:3**	17.77	0.10	3.092	1.101
C18:1 13c	66.10	0.08	7.622	**PUFA**		3.95	2.066	**C20:4**	18.17	0.18	2.755	4.441
C18:1 16t+14c	67.74	0.35	2.442					**SFA**		67.82	0.116	0.444
								MUFA		26.97	0.284	0.582
								PUFA		3.80	0.929	2.806

i: iso; ai: anteiso;t: *trans*; c: *cis*; NMID: non methylene interrupted diene; CLA: conjugated linoleic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. CV (Coefficient of variation) calculated with means and standard deviations from data (n=3). CV[1] calculated with means and standard deviations of the obtained data (n=3). CV[2] used in the inter-assay test calculated with the corresponding mean and standard deviation from CPSil-88 and VF23ms.

.Table 2. Fatty acid composition (g FA/ 100 g F) of oil samples (VF23ms).

		Fish		Safflower		Sunflower		Soya		Tung		Tonalin®	
Fatty acid	**Rt (min)**	**Mean**	**RSD**	**Mean**	**RSD**	**Mean**	**RSD**	**Mean**	**RSD**	**Mean**	**RSD**	**Mean**	**RSD**
C8	2,89			0,04	3,539								
C10	3,79	0,03	9,552	0,03	4,319								
C12	4,99	0,17	5,805	0,21	1,987								
C13	5,63	0,06	5,444										
C14	6,30	8,02	4,980	0,22	1,544	0,09	2,440	0,10	6,05	0,04	1,994		
C15	7,01	0,52	5,632	0,02	2,330	0,02	5,838						
C16	7,76	17,91	4,616	8,89	0,396	7,63	0,469	13,13	0,38	2,67	0,265	0,93	0,337
C17iso	7,96	0,52	3,572	0,06	2,693								
C16:1	8,16	5,59	4,527	0,10	0,629	0,12	0,490	0,12	1,67				
C17ai	8,26	0,45	4,197										
C18	9,37	4,09	4,811	3,13	0,159	4,37	1,184	3,83	0,63	3,03	0,085	3,04	0,262
C18:1 5t-9t	9,61	1,06	5,658										
C18:1 10t	9,63											0,04	4,377
C18:1 9c	9,76	15,64	4,668	15,33	0,068	30,73	0,861	28,38	0,97	7,54	0,226	14,30	0,160
C18:1 11c-15c	9,84	3,59	3,880	0,93	0,193	0,99	2,479	1,47	3,75	0,37	3,971	0,90	2,216
C18:2 t,t NMID	10,00											0,11	3,399
C18:2 9t,12t	10,09											0,09	6,535
C18:2 9c,13t	10,23	0,18	7,022	0,74	0,159	0,12	9,291	0,24	2,66	0,67	0,291	0,17	1,549
C18:2 9c,12t	10,24	0,19	5,157	0,66	0,317	0,06	1,077	0,19	4,76				
C18:2 11t,15c	10,31											0,16	1,122
C18:2 9c,12c	10,41	12,10	4,847	68,07	0,015	54,50	0,321	45,70	0,65	5,55	0,127	0,12	1,635
C20	10,86	0,181	7,019					0,28	1,40				
C18:3 9c,12c,15c	11,19	1,05	5,983	0,12	3,541	0,11	2,014	4,58	1,20	0,10	6,133		
C20:1 n9	11,20					0,16	0,677						
CLA 9c,11t	11,39											39,06	0,108
CLA 10t,12c	11,53											38,89	0,085
CLA c,c	11,63											1,07	0,666
C18:4	11,67	2,82	4,273	0,17	2,865								
CLA t,t	11,79											0,97	0,718
C20:2 c,c	13,01			0,25	0,415	0,65	1,403	0,36	1,09				
C20:4 AA	13,14	0,68	4,001										
CLnA 9c,11t,13t	13,96									72,44	0,088		
C20:5 EPA	14,02	9,88	4,452										
CLnA B	14,39									6,39	0,406		
C22:5 DPA	15,54	0,44	4,734										
C22:6 DHA	15,68	1,21	0,441										
SFA		31,913	2,302	12,57	0,316	12,11	0,623	17,34	0,41	5,76	0,176	3,96	0,279
MUFA		24,48	4,031	16,36	0,062	31,99	0,785	29,96	1,11	7,90	0,087	15,24	0,017
PUFA		28,55	4,448	70,13	0,016	55,71	0,310	51,22	0,72	86,15	0,103	80,64	0,026

i: iso; ai: anteiso;t: *trans*; c: *cis*; NMID: non methylene interrupted diene; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. CV (Coefficient of variation) calculated with means and standard deviations from data (n=3).

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Manuscript Draft

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Title: Major lipid classes separation of buttermilk, and cows, goats and ewes milk by HPLC-ELSD focused on the phospholipid fraction.

Article Type: Full Length Article

Keywords: Evaporative light-scattering detection (ELSD-HPLC), lipid class separation, milk phospholipids

Corresponding Author: Dr. JAVIER FONTECHA,

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COVER LETTER

Madrid-23-Octobre-2009

Dear Editor of Journal of Chromatography A

Attached please find the manuscript entitled: “Major lipid classes separation of buttermilk, and cows, goats and ewes milk by HPLC-ELSD focused on the phospholipid fraction (Rodríguez-Alcalá, L.M. and Fontecha, J.)

We confirm that the work presented in this manuscript is new and original and it is not under consideration elsewhere. Our institution agrees to the submission of this paper to the Journal of Chromatography A.

Sincerely,

Dr. Javier Fontecha.

Major lipid classes separation of buttermilk, and cows, goats and ewes milk by HPLC-ELSD focused on the phospholipid fraction.

Rodríguez-Alcalá, L.M. and Fontecha*, J.

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Keywords: Evaporative light-scattering detection (ELSD-HPLC), lipid class separation, milk phospholipids

Abstract

An improved HPLC-ELSD method have been developed for the analysis of the lipid classes of buttermilk and milk from different species, focused in the phospholipids fraction without a prior fractionation step and in a single run. The total lipid profile analysis showed the major and minor lipids compounds as cholesterol esters, triacylglycerides, cholesterol, diacylglycerides, free fatty acids, monoacylglycerides, and also the polar compounds as glucosylceramide, lactosylceramide, phosphatidyl-ethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomieline and lysophosphatidylcholine. The identification and quantification of the different compounds, using calibration curves made with individual standards and the low coefficients of variation obtained in the inter- and intra-assays, showed the suitability of the developed method. In this study, we optimized and validated a quantitative HPLC-ELSD method at a concentration level suitable for routine analysis of the major lipid classes in milk and dairy products.

1. Introduction

Milk fat have been shown to be an importance source of bioactive compounds claiming nutritional and functional benefits as ingredients of food products in order to protect and promote the consumers health [1,2]. The lipid phase in milk exists as an emulsion of oil droplets dispersed in the aqueous phase that takes the form of spheres (0.1–15 μm) surrounded by a 4- to 10-nm multilayer membrane composed primarily of triglycerides, proteins, and phospholipids, which is referred as milk fat globule membrane (MFGM) [3]. This multilayered chemical complex correspond to a quantitatively small lipid fraction but represent an interesting and valuable natural material with potential textural and bioactive properties [4,5], which could be used in many food applications because of the presence of polar lipids as phospholipids (PLs) and specific glycoproteins [4,6,7]. Some PLs have been associated as agents against colon cancer, gastrointestinal pathogens, Alzheimer's disease, depression, and stress as well as their amphiphile nature makes them suitable to be added into a variety of foods matrix. Rich sources of these polar lipids are the aqueous phases of milk fat obtained as a byproduct in the elaboration of butter known as buttermilk [8]. Buttermilk is a liquid product rich in proteins and phospholipids, from the MFGM (disrupted in the buttermaking) and whey [9] as well other water-soluble compounds of milk [10,11]. Due to its technological and biological properties, buttermilk could have a great impact as functional ingredient. PLs are present in all foodstuffs and, because of their emulsifying properties they can exert profound effects during food processing. PLs play an important role in governing the quality of dairy products during processing and they are important flavour precursors because of their high content of long-chain polyunsaturated fatty acids.

Due to FA profiling of lipids by GC does not allow correlating FA identification with their lipid class, traditionally, the separation of the lipids into the individual classes of origin (e.g., acylglicerols, phospholipids, fatty acids, cholestryl esters, etc), was achieved by silica gel column chromatography, preparative TLC or by lipid fractionation using SPE [12]. Nevertheless these approaches resulting in poor reproducibility and produce significant losses of FA as they can result in low and variable lipid fraction recoveries or oxidation of PUFAs in the presence of certain brands of silica gel [13].

The introduction of the ELSD brought a major advance in the detection separation and quantification of lipid compounds [14-18]. Being sensitive just to the mass of vaporized analyte, the ELSD is not limited by the solvent flow rate or ambient temperature that allows for a significant improvement in analysis time, good sensitivity level and, moreover, it is compatible with all gradient elution.

The aim of the present study was to develop a gradient solvent system for HPLC-ELSD able to separate and quantify the major and minor lipid classes present in powder buttermilk, with especial interest in the phospholipid fraction, without a prior fractionation step and in a single run. The content and distribution of lipid classes and phospholipids in cows, ewes and goats milk have been also analyzed.

2. Experimental

2.1. Chemicals

The solvents chloroform, methanol, isooctane and isopropanol, were HPLC grade, purchased to LABSCAN (Dublin, Ireland). Triethylamine (99.5%) and formic acid (98%) were purchased to Sigma (Bellefonte, PA, USA). Cholesterol, cholesteryl ester, tritridecanoin, monoacylglicerol, dioleinglicerol, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin (SM) were used as standards (Sigma, Bellefonte, PA, USA).

2.2. Extraction of the lipid fraction of the samples

The extraction of the lipid fraction of powdered buttermilk (kindly donated by Reny Picot, Navia, Asturias, Spain), obtained as a sub-product in the industrial manufacture of butter oil, was carried out according to the Folch method [19] to ensure the completed extraction of the whole lipid classes. Briefly, 4 g of powder sample was thoroughly dissolved in 10 ml distilled water, and 75 mL chloroform/methanol (2:1) was added, vortex during 2 minutes and then slightly shaking during 1h at 4ºC. After centrifugation 5 minutes at 5000 rpm, the lower chloroform layer was released and the process repeated adding 50 mL of chloroform to the upper (methanol) phase. The two chloroform phases were pooled and evaporated using a rotary vacuum evaporator at 35ºC. Separated lipids were stored in amber vials, exposed to a stream of N2, and frozen at -20°C until analysis. Same procedure was used for extraction of milk fat fraction

from 3 ml of whole untreated milks. Each extraction was performed 6 times and injected twice.

2.3. Chromatographic conditions

The neutral and polar lipid classes were separated by different solvent mixtures and gradient systems. Details of the mobile phase composition and gradient elution profile are given in Table 1. Triethylamine was added to the solvents for HPLC of polar lipids in order to improve peak shape and resolution. Furthermore, triethylamine also improved the reconditioning of the column and made sure that no lipids remained adsorbed in the column after each analysis.

Pure lipid standards were chromatographed individually to confirm retention times and purity. All standards were identified and quantified using calibration curves.

2.3.1. HPLC-ELSD analysis

Separation of lipid classes was accomplished in a HPLC (Shimadzu Vp Series, Duisburg, F.R. Germany) coupled with an ELSD detector (SEDERE. SEDEX 85 model, Alfortville Cedex, FRANCE) using filtered air as the nebulizing gas at a pressure of 3.5 bar, at 60 ºC and the gain was set at 3. A 250mm x 4.5 mm Zorvax Rx-SIL column (Agilent Tecnhnologies, Palo Alto, CA, USA) with 5-μm particle diameter and a precolumn with the same packing was used.

Samples were dissolved in chloroform prior to analysis, at a concentration 0.1 mg/ml. The injection volume was 10 μL and the column was equilibrated at 40ºC.

Injections were performed at least by triplicate and in different days with a fresh preparation of samples and solvents.

2.4. Statistics

Statistics of the compositional data of buttermilks and milk fats were performed with the aid of the SPSS package (SPPS package (SPSS 17 for Windows, SPPS Inc., Chicago, IL, USA). Intra and inter-assay precision was assessed by the relative standard deviation (RSD) of 3 replicates per sample and carried out by the same operator. Differences among composition of cow, goat and ewes milks were analyzed using the ANOVA procedure with the Bonferroni´s comparison test of mean values (n=3 for each sample).

3. RESULTS

Lipid classes are defined by the polar head-group nature. Within a single lipid class, different molecular species may occur. Since the introduction of the ELSD-HPLC, new methods have been published for the separation of neutral and/or polar lipids. In the present study, we achieved for milk fat, not only the separation of lipid classes but the phospholipids in a qualitative and quantitative conditions in the same run and without prior lipid fractionation. As a lipid class profiling procedure aims at quantifying lipid class distribution, elution of all molecular species of a defined class in a single and sharp peak will allow the most accurate results.

163

164 *3.1. Validation of the method conditions*

165 Calibration curves for detector response versus mass of lipid injected were obtained
166 by applying the power model [20]. Serial diluted concentrations of pure PC at 2.3 to
167 15.2 μg/mL, PI at 2.3 to 15.3 μg/mL, PS at 2.2 to 14.9 μg/mL, PE at 2.2 to 15 μg/mL,
168 SM at 2.3 to 15.6 μg/mL and lysophosphatidilcholine (LPC) 2.2 to15 μg/mL, were
169 injected by triplicate. The nonlinear response of the detector to lipid mass, which is
170 characteristic of mass detection by ELSD [21], was well fit by power model equations
171 and that the assayed conditions are suitable for the assessment of phospholipids: $y = 34310x^{1,2604}$, $R^2 = 0,994$; $y = 11748x^{1,2647}$, $R^2 = 0,994$; $y = 31405x^{1,2618}$, $R^2 = 0,995$; $y = 22097x^{1,2311}$, $R^2 = 0,990$ and $y = 31026x^{1,0745}$, $R^2 = 0,994$, respectively. The mass of
174 each PLs in buttermilk and milk fat lipid extracts was obtained from the corresponding
175 fitted calibration curves.

176 Sensitivity (S), limit of detection (LOD) and limit of quantification (LOQ) were also
177 evaluated in the current method (Table 2). According to IUPAC [22], sensitivity is
178 defined as the slope of a calibration curve in linear curve. However, as ELSD detectors
179 have non-linear response, in this situation, S can be calculated as derivative of the
180 power model with respect to the injected amount, giving the following expression:
181 $S=Abx^{b-1}$, where A and b are terms obtained from the power model and x is the
182 concentration of the injected amount. Therefore, the S parameter was calculated for all
183 the PLs examined at the lowest injected amount. The LOD and LOQ values were
184 calculated as $LOD=3.3*\sigma/S$ and $LOQ=10*\sigma/S$ where σ is the standard deviation of the
185 response and S the sensitivity. As can be seen in table 2, the lowest values for these
186 parameters (S, LOD and LOQ) corresponded to PI while the SM presented the highest

187 values. The detection limit (peak/ noise) for neutral lipids at was approximately 30 ng.
188 The method described here, therefore, is sensitive enough to quantify the PL levels
189 present in dairy products and also in biological systems.

190 The accuracy of the proposed method was tested by analyzing the coefficients of
191 variations (CV) for each sample of the intra-assay (measures in a same day) and inter-
192 assay (different days) (Table 3), and for all analyzed compounds showed values under
193 10% of acceptance for chromatographic analysis. The excellent precision in terms of
194 repeatability of peak areas, retention times and detector responses indicate that this
195 method is suitable for a precise determination of lipid classes and specially
196 phospholipids.

197

198 *3.2. Composition and distribution of neutral lipids*

199

200 Figures 1 and 2 show the chromatographic profile of buttermilk sample (BM) and
201 milk fat (MF) sample respectively, obtained by HPLC-ELSD. The peak appearance and
202 the elution position upon HPLC were highly reproducible for most neutral and polar
203 lipid classes. The peak areas for the lipid classes in BM and MF from cows, ewes and
204 goats milk were converted into absolute amounts using the linear regression analysis of
205 the calibration curves generated from standard lipids. The percentage lipid composition
206 of BM and MF are shown in Tables 3 and 4. As expected the most abundant neutral
207 lipid class was dominated by TAG, which represented nearly 54.5% for BM (Table 3)
208 and 97.5-98% for MF (Table 4) of total lipids and eluted divided in two peaks attributed
209 to long and short chain TAG moieties. The use of isooctane as the main solvent in the

mobile phase at the beginning of the analysis allows a rapid elution and good resolution of the less polar lipid classes (i.e. CE and TG). With this procedure it is solved one of most complicated challenges for separation of lipid classes in dairy products which is the separation of TAG from CE and also avoiding the TAG saturation of the ELSD response. The rest of neutral lipids constituted by DAG, Chol and FFA that coeluted in one peak, plus MAG accounted around 12% for BM (Table 3) and 1.8 % for MF (Table 4). After neutral lipids elution, this method allowed a baseline separation of all investigated polar lipids, with symmetrical peaks, covering a polarity range between GluCer, LacCer and the phospholipids in one operation sequence without previous fractionation or purification needed. In BM, the total polar lipid concentration was about 30% which is in the same range than were found by other authors on commercial powder BM [11]. Nevertheless big variations of the polar lipid content have been reported for buttermilk, ranging 4.5 to 33 % of total lipid [23, 24]. In the conditions assayed the predominant phospholipid was PC with 35% followed by PE and PS with about 20% and the SM content, calculated from the sum of the two peaks, was 17%. Buttermilk phospholipids had distribution and concentrations as from other previous investigations [25],[8] where the reported amounts ranged 15-42 % for PE, 0.3-9 % for PI, 3-10 % for PS, 19-30 % for PC and 12-29 % for SM [8,25,26]. Although there is scarce information about the distribution of neutral lipids in powdered buttermilk, Morin et al, [27] observed a decreasing proportion of some PLs as PE after spray-drying of buttermilk and a proportional increase of the PC and SM. They report that a loss of a major phospholipid would be an indication of a loss of MFGM fragments during processing. Those results agree with the high amount of PC found in this study.

In MF obtained from raw cows, goats and ewes milk, significant differences were found for the total polar lipid content (Table 4). Goats milk fat showed higher values

than cows and ewes milk for total polar lipid content (0.65 vs. 0.36 and 0.38 respectively) although great variations (between 0.3 and 1.2 %) have been also reported for total phospholipids from raw milk [23, 24]. In a recent study [28] higher values have been reported for the total phospholipids content found in cows, ewes and goats milk (0.8, 1.0 and 1.0 % respectively). In this study, the predominant phospholipid was PE followed by PC and SM in agreement with the previously published data. Elsewhere have been described that mammalian milks have a similar phospholipids distribution [29], although contents can differ due to genetics, state of lactaction and feed factors [30, 31] as can being observed in the cow, goat and ewes milk samples.

3.3 Concluding remarks

HPLC-ELSD is undoubtedly an important element of the techniques which are now rapidly developing in lipid analysis. Correct information about the factors that govern the retention and selectivity of individual lipid compounds will help to increase the efficiency and credibility of the analytical procedures.

The overall results of this work confirm that the HPLC-ELSD developed method yields a simple, sensitive, stable, and comprehensive quantification of all the major lipid classes, focus on the phospholipid fraction. The developed gradient solvent system for HPLC-ELSD was able to separate with high reproducibility and quantify with accuracy the major and minor lipid classes, with especial interest in the phospholipid fraction, present in powder buttermilk, and milk from different species, without a prior

fractionation step and in a single run. Therefore this method can be recommended for the routine analysis of fat fraction of milk and dairy products.

Acknowledgments

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Figure captions

Fig. 1. Separation of the lipid classes of powder buttermilk fat samples by HPLC-ELSD. The individual lipids of these samples are quantitated in Table 3. CE: cholesteryl ester; TAG: triacylglycerides; DG: diglycerides; CH: cholesterol. FFA: free fatty acids; GluCcer: glucosylceramide; LaCcer: lactosylceramide; PA: phosphatidic acid; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin; LPC: lysophosphatidilcholine.

Fig. 2. Separation of the lipid classes of cows milk fat sample by HPLC-ELSD. The individual lipids of this sample is quantitated in Table 3. CE: cholesteryl ester; TAG: triacylglycerides; DG: diglycerides; CH: cholesterol. FFA: free fatty acids; Gluccer: glucosylceramide; Laccer: lactosylceramide; PA: phosphatidic acid; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin.

Figure

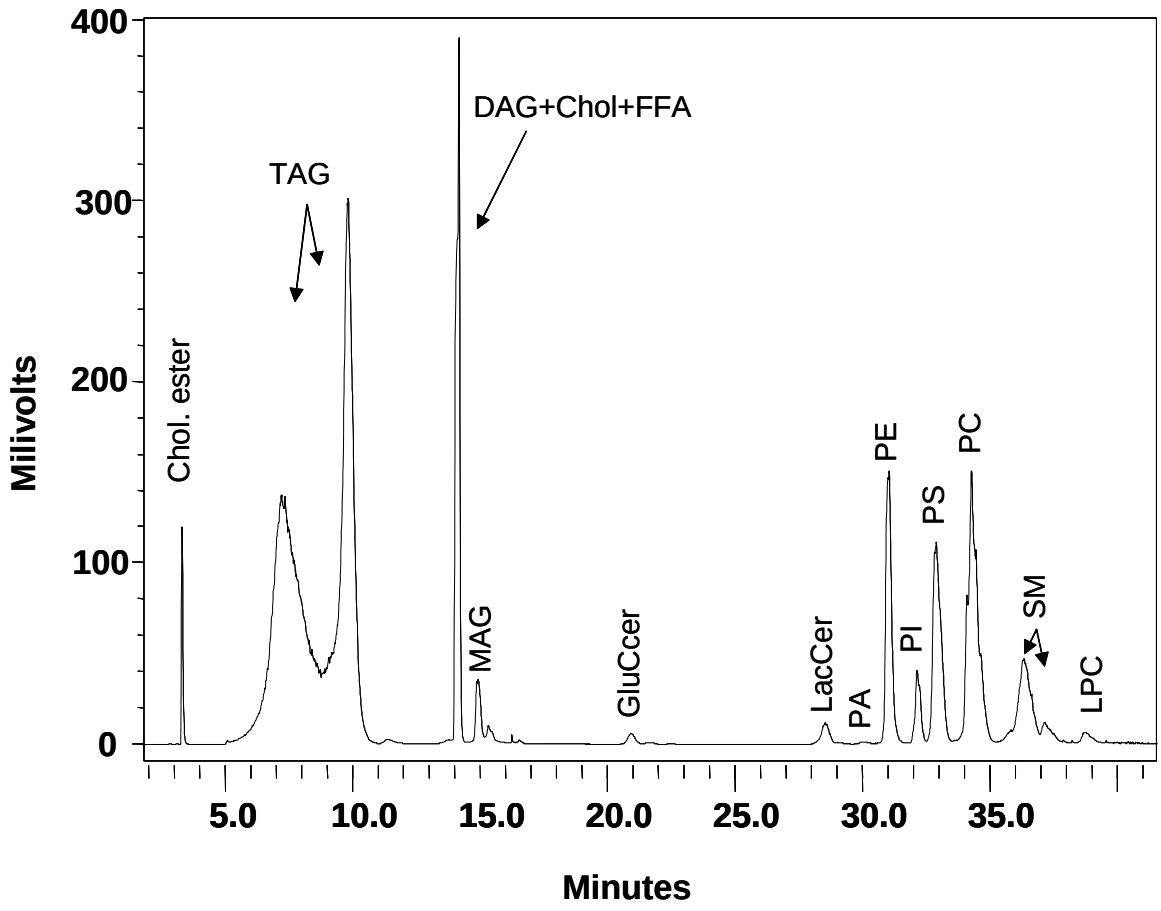

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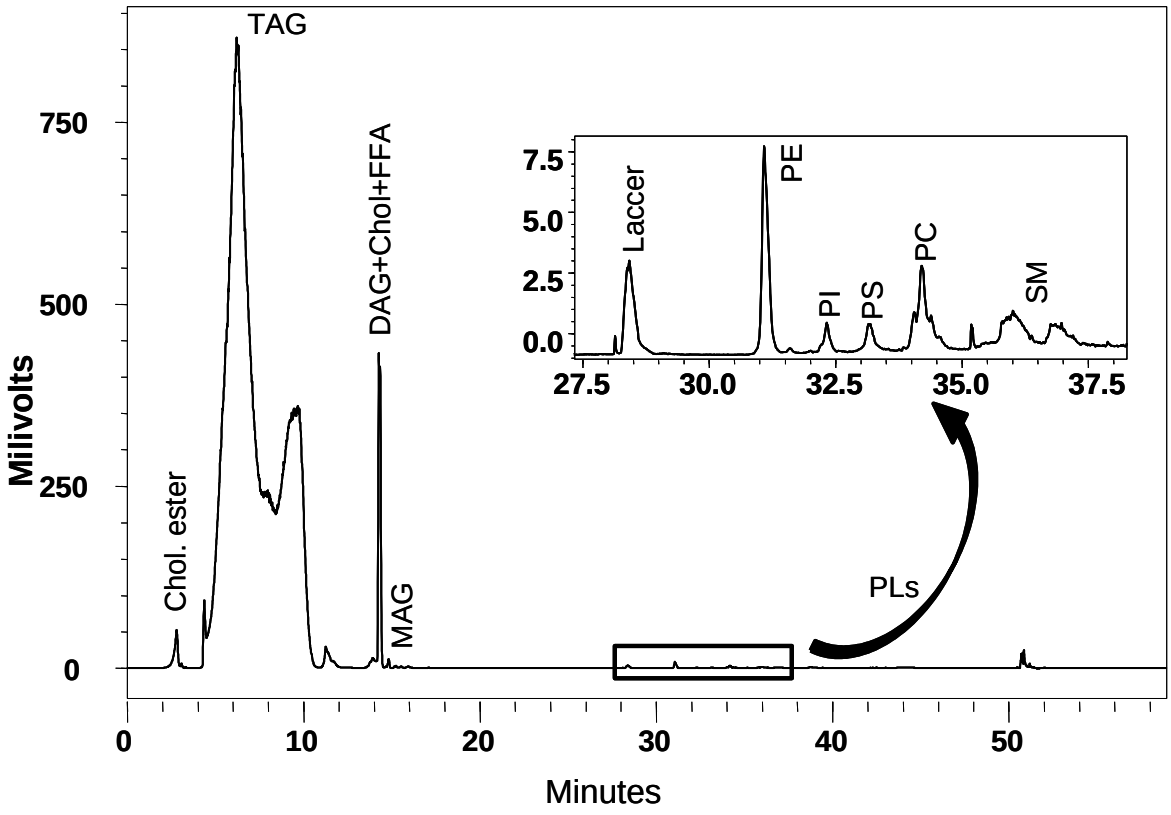
Figure



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Tables

Table 1

Solvent gradient elution system required for the elution of lipid classes and reactivation of the column

	Percent Solvent				
Time (min)	**A**	**B**	**C**	**D**	**Flow (mL/min)**
0	0	0	100	0	1
3.5	0	0	100	0	1
19	100	0	0	0	1
21	100	0	0	0	1
41	0	100	0	0	1
42	100	0	0	0	1
42.01	100	0	0	0	1.5
47	100	0	0	0	1.5
47.01	0	0	0	100	1.5
48.99	0	0	0	100	1.5
49	0	0	100	0	1.5
54	0	0	100	0	1.5
59	0	0	100	0	1
59.01	0	0	100	0	0

A: Chloroform/Methanol/Water (1M formic acid; TEA. ph=3). 87.5:12:0.5 (v/v/v).
B: Chloroform/Methanol/Water (1M formic acid; TEA. ph=3). 28:60:12 (v/v/v).
C: Isooctane/THF. 99:1 (v/v).
D: 2-propanol

Table 2

Sensitivity (S), limit of detection (LOD) and limit of quantification (LOQ) for standards phospholipids in the assayed method.

Compound	**Amount (µg)**	**S (mV/µg)**	**LOD (µg)**	**LOQ (µg)**
PE	2.205	20	0.558	1.690
PI	2.295	2	0.299	0.906
PS	2.228	13	0.321	0.974
PC	2.273	16	0.380	1.151
SM	2.340	20	0.751	2.277
LPC	2.250	23	0.400	1.214

PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin; LPC: lysophosphatidylcholine.

Table 3

Mean values (percentage) and coefficient of variation (CV) of the lipid composition of lipid classes content of powder buttermilk determined by HPLC-ELSD.

Lipid classes (%)	Mean	CV intra-assay	CV intra-assay	CV inter-assay
		Day 1	Day 2	
Neutral Lipids	68.28	0.435	0.136	1.851
Polar Lipids	31.72	0.986	0.278	3.985
Neutral lipid fraction (% of total lipids)				
CE	1.50	3.406	2.758	5.468
TAG	54.55	0.357	0.064	3.361
Chol+DAG+FFA	10.51	1.320	0.459	4.749
MAG	1.73	1.028	2.068	1.562
Phospholipid fraction (% of polar lipids)				
Gluccer	1.22	1.796	5.850	4.616
Laccer	2.37	1.269	1.936	1.496
PA	0.30	0.340	0.071	2.132
PE	19.80	0.759	0.363	0.489
PI	4.93	2.414	0.549	1.484
PS	20.60	0.628	0.350	0.416
PC	33.91	0.289	0.204	0.204
SM	16.87	1.722	1.781	1.455

CE: Cholesteryl ester; TAG: triacylglycerides; DG: diglycerides; CH: cholesterol. FFA: free fatty acids; Gluccer: glucosylceramide; Laccer: lactosylceramide; Neutral lipids: CE+TAG+DAG+Chol+FFA+MAG; Polar lipids: Gluccer+Laccer+Phospholipids; PA: phosphatidic acid; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin.

Table 4

Mean values and coefficient of variation (CV) of the lipid composition of lipid classes content of cows, goats and ewes milk fat determined by HPLC-ELSD.

	Cow		Goat		Ewe	
Lipids	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**
Neutral lipids	99.64[a]	0.014	99.35[a]	0.017	99.62[a]	0.015
Polar lipids	0.36[a]	3.939	0.65[b]	2.659	0.38[a]	3.793
Neutral lipid classes (% of total lipids)						
CE	0.04[a]	2.697	0.04[a]	2.463	0.02[a]	9.318
TAG	97.75[a]	0.097	97.32[a]	0.063	98.11[a]	0.072
Chol+DAG+FFA	1.81[a]	6.324	1.89[a]	2.930	1.45[b]	4.220
MAG	0.04[a]	2.989	0.10[a]	9.959	0.03[a]	3.302
Phospholipid fraction (% of polar lipids)						
Laccer	5.10[a]	13.420	7.57[b]	6.320	4.98[a]	4.110
PE	36.58[a]	0.570	29.17[b]	8.990	32.65[ab]	0.640
PI	6.18[a]	4.860	5.77[a]	3.690	4.16[b]	6.880
PS	7.28[a]	5.710	7.65[a]	8.650	4.96[b]	4.360
PC	24.60[a]	4.300	26.25[b]	3.690	27.21[b]	5.070
SM	20.25[a]	6.440	23.24[b]	6.510	26.05[b]	5.460

CE: Cholesteryl ester; TAG: triacylglycerides; DG: diglycerides; Chol: cholesterol; FFA: free fatty acids; LacCer: lactosylceramide; Neutral lipids: CE+TAG+DAG+Chol+FFA+MAG; Polar lipids: LacCer+phospholipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin. Different superscripts letters are for significative differences among species ($p<0.05$).

Reviewer Suggestions

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3.2. Estudio de la mejora nutricional del perfil lipídico de la leche mediante la incorporación a la dieta de rumiantes de suplementos ricos en PUFA, o mediante empleo de bacterias lácticas con capacidad para transformar el ácido linoleico en isómeros del ácido linoleico conjugado (CLA).

Draft Manuscript for Review

Influence of feeding linseed at different levels on fatty acid profile focused on the CLA isomers composition of goat milk.

Journal:	*European Food Research and Technology*
Manuscript ID:	Draft
Manuscript Type:	Original paper
Date Submitted by the Author:	
Complete List of Authors:	CALVO, MARIA; CSIC, DAIRY PRODUCTS RODRIGUEZ-ALCALA, LUIS; CSIC, DAIRY PRODUCTS KIVES, JULIANA; FORLASA, R+D ROMERO, JESUS; Laboratorio de Lactología y Sanidad Animal FONTECHA, JAVIER; CSIC, DAIRY PRODUCTS
Keywords:	goat milk, Conjugated linoleic acid, linseed, health-promoting index (HPI), fatty acids

Influence of feeding linseed at different levels on fatty acid profile focused on the CLA isomers composition of goat milk.

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Abstract

In this study the analysis of milk from a number of goats herds from Castilla-La Mancha (Spain) revealed notables differences in the fatty acid profile, particularly the total CLA content varied from 0.3 to 1.2 g/100 g fat. A feeding trial was carried out with the aim to enhance the CLA contents in goats´ milk fat under field conditions by dietary means. Thus, a commercial supplement enriched in linseed (SEL) was incorporated at three different concentration levels into the goats' diet and the fatty acid profile and CLA isomers content in milk fat was thoroughly monitored. Among the SEL doses assayed, 0.7 kg/animal/day provided the best results ($P<0.05$). A noticeable decrease in saturated fatty acid level was detected in goats fed with SEL which was highly correlated to the lower concentrations of C12:0, C14:0 and C16:0. Concentrations of C18:1 *trans*-11 and C18:3n-3 were markedly enhanced during the period of supplementation. The principal CLA isomer *cis*-9, *trans*-11 C18:2 accounting for as much as 90% of the total CLA. Levels of other minor CLA isomers (mainly 11-13 *trans-trans* and *trans-cis* geometric isomers as well as *trans*-7, *cis*-9) were also enhanced as consequence of lipid supplementation.

Keywords: Conjugated linoleic acid; goat milk; linseed; health-promoting index (HPI), fatty acids.

Abbreviations: PUFA= polyunsaturated fatty acids; **MUFA** = momounsaturated fatty acids **SFA=** saturated fatty acids; **SEL** = supplement enriched in linseed.

Introduction

Historically, the goal of the agricultural research has been to increase yield in productive efficiency, with little focus given to improving the nutrient profile and manufacturing properties of the food products. More recently, nutritional quality has become increasingly important in foods choices because of consumer awareness of the link between diet and health [1].

Dairy foods contain components that may provide benefits to human health as bioactive components have been discovered in milk [2-5]. In particular, there is a wide range of bioactive components in milk fat that have been identified as possessing effects of potential benefit to chronic human diseases prevention and health maintenance. Much attention has been directed toward conjugated linoleic acid (CLA) since the discovery of its anticarcinogenic properties three decades ago [6]. Recently, some authors [7,8] have reviewed the numerous biological activities reported over past few years confirming that individual CLA isomers present in milk fat have a high health promoting potential. Milk from small ruminants and dairy products were found to be the richest sources of CLA [8,9] and provide approximately 75% of our dietary intake of CLA. The isomer cis-9, trans-11 CLA, also known as rumenic acid (RA), is the one that naturally predominates in foods obtained from ruminants [10]. RA represents 75 to 90% of the total CLA in milk fat, and its presence is related to the biohydrogenation of polyunsaturated fatty acids that takes place in the rumen [11-13].

It is widely recognized that diet plays a major role on modulating the fatty acid composition of ruminant milk [14,15] and hence it represents a practical tool to alter the yield and the fatty acid composition of milk fat. The aspects of the nature and composition of the animal's diet that have an influence on the fat content of its milk and its composition has been recently reviewed [16]. Among animal feeding strategies for CLA enrichment of milk, those diets with

linseed supplements rich in PUFA that provide lipid substrates for the production of rumenic acid or *trans*-vaccenic acid have proved to be the most effective [17,18]. In addition to enhancing CLA content, the dietary changes with linseed also result in milk fat containing a lower proportion of saturated FA and greater amounts of mono unsaturated FA and PUFA.

As some authors have pointed [19,20], the number of studies on relationship between diet and CLA concentration in sheep and goat milk fat is rather limited compared to cows. Despite of the convincing evidences for the special value of goat milk in nutrition and well-being [21], its unique qualities are barely exploited. Furthermore, although there are several papers reporting data on total CLA [19,22,23] in goat milk, there is limited information about minor isomers.

The aim of this study was firstly to determine the CLA and n-3 content and fatty acid profile of goat milk fat from a wide number of dairy farms located in Castilla-La Mancha (Spain). Afterwards, three of the analysed goat's herds were chosen to perform a feeding trial designed to modify the milk fat towards healthier fatty acids composition. A commercial supplement enriched in linseed (SEL) at three different levels was incorporated to the herd's diet. Changes in the fatty acid profile of goats' milk fat as well as CLA isomer distribution were analysed.

Materiales and Methods

Livestock exploitations

Milk samples were collected from fourteen different farms (each one with a minimum of 400 heads) located in Castilla-La Mancha (Spain) to ensure a variety of milk compositions. During a 3-wk period (from April to May), samples were taken, twice at week, from the storage tanks containing milk from the whole herd. Freshly drawn milks were shipped to the laboratory in isothermal containers. Milk fat from all herds was evaluated with regard to chemical composition and the fatty acid profile was also thoroughly monitored.

Feeding experiments

Once FA profile of goat milk from different studied areas was characterized, the impact of a diet supplemented with linseed on FA composition of milk fat was evaluated in three herds of goats Murciano-granadina crossbreed. From each of the three selected herds 48 goats were separated and fed a SEL diet. Milk samples from the rest of the herd (which received control diet) was also collected and analysed to obtain an external control.

The study was conducted over a 10-wk period (from September to November). Bulk milk samples were collected twice at week during the milking period from all herds. For the first 3-wks the animals were gradually adapted to SEL. This was by replacing conventional diet with increasing amounts of a commercial supplement enriched in extruded linseed (SEL diet, Cargill España S.A) until the desired dose was reached. Once this intake was stabilised, animals were kept under experimental conditions for the following 4-wks. According to the PUFA total content of the analyzed milks with special reference to the CLA content, the

fourteen farms studied were grouped in high, medium and low content, denoted as A, B and C respectively. One farm of each of the three groups A, B and C was selected and supplemented with 0.3, 0.5 and 0.7 kg SEL per animal per day, respectively. These SEL doses correspond to 17%, 24% and 39 % of the total daily ration. The ingredient and chemical composition of the control and SEL diets are shown in Table 1 and 2. Finally, during the last 3 weeks the supplement was removed. Changes in milk yield as well as in body conditions of specimens under study were also evaluated.

Milk chemical analyses

Fat, protein, lactose and total solids in milk were measured with a MilkoScan FT-6000 (Foss Electric, Barcelona, Spain).

Lipid extraction and fatty acid derivatization

Fat separation was carried out according to Luna et al [24]. Thus, the refrigerated raw milk sample (100 millilitres) was tempered at 20°C for 20 min, and centrifuged at 17,800 x g for 30 min at the same temperature in a Beckman (Fullerton, CA) J2-MC centrifuge. The fat layer was removed, transferred to microtubes, and centrifuged at 19,300 x g for 20 min at room temperature. After the second centrifugation, the top layer was removed for analysis. Separated lipids were stored in amber vials, exposed to a stream of N_2, and frozen at –20°C until analysis. In all cases, fatty acids methyl esters of milk fat (FAME) were freshly prepared by base-catalyzed methanolysis of the glycerides using KOH in methanol as described in ISO-IDF [25].

GC-FID analyses

FAME contents were determined by using two different chromatographic systems. Due to the high number of samples analysed during the characterization of herds from Castilla-La Mancha (Spain), a fast GC analysis on a VF-23ms, fused-silica capillary column (30 m x 0.25 mm i.d.x 0.25 µm film thickness, Varian, Middelburg, Netherlands), was used. The FAME were analysed on a Clarus® 500 chromatograph from Perkin Elmer. The column was held at 120ºC for 1 min after injection, temperature-programmed at 10ºC/min to 140ºC, then temperature-programmed at 15ºC/min to 180ºC and last ramp at 5ºC to 240ºC held there for 3 min. Helium was the carrier gas with a column inlet pressure set at 15 psig and a split ratio of 1:20. The injection volume was 0.5 µl. Total run time was 15 min.

On the other hand, for a more exhaustive identification of the fatty acids and to achieve a more complete lipid profile, a second chromatographic system besides the former system described above, was used during the feeding trial. Thus, FAME were separated by using a CP-Sil 88 fused-silica capillary column (100 m x 0.25 mm i.d.x 0.2 µm film thickness, Chrompack, Middelburg, Netherlands) on an Agilent chromatograph (model 6890N, Palo Alto, CA, USA) equipped with a flame ionization detector. The column was held at 100 ºC for 1 min after injection, temperature-programmed at 7°C/min to 170ºC, held there for 55 min, then temperature-programmed at 10°C/min to 230ºC and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 psig and a split ratio of 1:20. The injection volume was 0.2 µl. Total run time was of 105 min.

For FAME determination and quantification, an anhydrous milk fat (reference material BCR-164) (Fedelco Inc., Madrid, Spain) and tritridecanoine as internal standard (Sigma, St. Louis, MO) were employed.

Ag+-HPLC.

Ag^+ HPLC separation of CLA methyl esters was carried out using a HPLC chromatograph (Shimadzu Vp Series, Duisburg, F.R. Germany) equipped with a photodiode array detector operated at a wavelength of 19-300 nm and measuring CLA at 234 nm. A ChomomSpher 5 Lipids analytical column (4,6 mm i.d. x 250 mm stainless steel; 5 µm particle size; Varian) for separation of CLA isomers, was used. The mobile phase was 0,1 % acetonitrile in hexane and operated isocratically at a flow rate of 1 mL/min. The flow was initiated 0.5h prior to the sample injection and the injection volume was 10 µl. For identification of CLA isomers a mixture and pure CLA isomer methyl esters were pursached from Nu-Check-Prep. Inc. (Elysian, MN).

Health–promoting index (HPI)

Heath-Promoting Index (HPI) was calculated according to Chen et al., [26] as follows:

$$HPI = [\Sigma MUFA + \Sigma PUFA] / (C12:0 + 4 \times C14:0 + C16:0)$$

Statistical Analysis

Data are expressed as means ± SD. Student's paired t test was used to compare milk FA composition from goats fed control diet and supplemented diet, for each SEL dose tested. The effect of three different SEL doses on milk FA composition was evaluated by means one-way analysis of variance (ANOVA). All statistical analysis were performed using the SPSS package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL). The criterion for significance was $P < 0.05$ for all comparisons

Results and Discussion

Livestock exploitations

Descriptive data on composition of milk fat can be important for studies if the bulk raw milk is going to be used to manufacture cheese and other dairy products, as occurs in Castilla-La Mancha (Spain). Particularly, the possible variability of total PUFA and CLA content in milk fat from different dairy farms (under semi-extensive livestock system) in this Spanish region was evaluated since to date there are only few data on this topic. Data of compositional analysis as well as lipid profile from all herds are summarized in Table 3. Average of mean values of all herds is also presented. Regarding the compositional analysis, no significant variations among herds were found and the mean values were according to those previously reported for this goat breed [27]. Total fat contents found were around 5% for the majority of goats' herds, and only H5 and H11 reached 6% of fat.

However, the herds showed clear differences with regards the fatty acid composition of milk fat. Moreover other factors affecting milk composition are the breed, individuals, parity, season, environmental conditions, locality, and stage of lactation and health status of the udder [20].

With respect to the CLA content, a great variability among goat's herds was found and three groups of samples were distinguished, showing mean levels of CLA around of 1.1, 0.6, and 0.4%, denoted as groups A, B and C respectively corresponding as high, medium and low level. With the exception of H10 and H13, all goat herds belong to Murciano-Granadina breed, a goat variety well adapted to semiarid conditions [28]. The above mentioned herds

showed the lowest levels of total therefore this might suggest that breed could have some influence, although further research is required.

Feeding experiment

Among the exploitations previously analysed, three goat's farms (one from each group) were selected to carry out the supplementation trial. Herds A, B and C were fed a SEL dose of 0.3, 0.5 and 0.7 kg SEL/animal/day, respectively, in order to study the SEL dose effect and also with the objective to reach comparable CLA and PUFA content in the milk fatty acid content from all the farms.

Global composition

The effect of dietary supplement concentration on gross milk composition was evaluated and the results obtained during 30 days of assay are shown in Table 4. Goat's herds fed control diet showed differences, mainly in fat content, likely attributable to different management regimes as discussed in the previous section and to the stage of lactation. An increase in fat content (around 1 %) was found in all herds throughout the assay as consequence of stage of lactation. Nevertheless, this increase was slightly retarded in herds B and C by the incorporation of linseed supplement (either 0.5 or 0.7 kg/animal/day).

For the same herd, no significant differences were found for global composition of control milk and the milk from goats fed linseed supplement diet. Although it has been reported that milk fat content does not decrease with almost all studied fat supplements in goats [13], in this study a slight decrease in fat content but not significant was observed. The effect of SEL incorporation into the diet was minimal on the other compositional parameters ($p<0.05$),

whose levels remained almost invariable in all treatments throughout the trial period. Similar results were described by Luna et al., [29] with diet enriched in linseed and sunflower oil. Sanz Sampelayo et al., [30] working with the same breed, also reported that concentrations of dry matter, fat, protein and lactose were not affected by the level of fat (rich in PUFAs) added to the diet. In goats, the energy balance of the animal is considered to be the most important factor determining milk fat as well as protein content [31].

On the other hand, milk yields from all herds were recorded daily during the entire period of the study. The mean daily milk yield per animal was about 2 liters and these volumes decreased around 5-10 % during the feeding assay in all herds and at all doses of SEL assayed. Nevertheless, no changes in body condition of the experimental animals were found.

Fatty acid profile

The FA profile in milk fat from the different herds was monitored by GC-FID over the trial period. Table 5 summarizes the results obtained after 30 days of feeding assay. As expected milk FA content was altered as consequence of the SEL dose employed. Except for 0.3 kg SEL dose, which not caused noticeable changes in lipid profile as occurred in global composition, the other SEL doses assayed resulted in milk fat containing a lower proportion of SFA and greater amounts of PUFA. Similar results were also reported in Granadina goats fed protected fat rich in PUFAs of marine origin [30]. On the other hand, a significant alteration (P<0,05) in MUFAs proportion was only found in herd C (higher SEL dose).

The noticeable decrease (P<0.05) in SFA levels detected in goats fed with SEL (0.5 and 0.7 kg) was highly correlated to the lower concentrations (P<0.05) of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) fatty acids found. Thus, the sum of these fatty acids in herd C is ~ 6% lower (39% *vs*-33%) than in control diet milk fat (Table 5).

Goats fed with 0.5 and 0.7 kg SEL supplement showed higher levels of PUFAs than did goats fed control diet ($P<0.05$). This increase was particularly positive at 0.7 kg SEL dose (3.7% for the control diet and 6% for supplemented diet). Linoleic acid (C18:2*n*-6) was the main PUFA found in all herds and although its concentration decreased by adding SEL, this variation was not significant (Table 5). Similar results were reported in goats fed rolled canola seeds [32] or diets enriched with three different vegetable oils [33]. Particularly remarkable ($P<0.05$) was the rise in the 18:2 *trans*-11, *cis*-15 level in the milk fat of goats fed SEL. This isomer was first recognized as the main intermediate in the ruminal hydrogenation of α-linolenic acid *in vitro* and has also been confirmed recently *in vivo* (Loor et al., 2002) as the major 18:2 isomer produced during hydrogenation of α-linolenic acid in the rumen. Likewise, significant increase in linolenic acid (C18:3*n*-3) was also observed during the period of supplementation. Accordingly to Luna et al., [17] the process of biohydrogenation in the rumen seemed to be limited, because noticeable amounts of α-linolenic acid contents escaped hydrogenation and increased the amounts of this FA in the milk fat of herds fed SEL.

Control groups in herds A and B showed similar CLA percentage of total FA (~1%) meanwhile in herd C was lower (0,72%). An important increase in total CLA ($P<0.05$) was observed groups B and C during the supplementation period (Table 5). Figure 1 shows the temporal pattern development of total CLA concentration measured during 30 days in the three groups of herds studied. Plot reflects the 10 days of adaptation period to dietary treatments, characterised by a progressive increase in CLA concentration until the highest value was reached. Total CLA remained elevated during the weeks of SEL enriched feeding. After SEL was eliminated from diet, CLA content dropped to its original values. As can be seen the level of CLA was altered by feed, and this alteration depend on the SEL dose. Thus, at dose 0.3 kg SEL no significant differences between control and supplemented milk were detected. However, when 0.5 kg and 0.7 kg of SEL were incorporated into the diet, notable

increases in CLA content were attained (2-fold and 2.4-fold respectively) and although the principal isomer was C18:2 *cis*-9, *trans*-11 (RA), accounting for as much as 90% of total CLA, other minor isomers can also play an important biological role. Therefore the CLA isomers distribution was determined by Ag+-HPLC (Table 6). These results revealed that C18:2 *cis*-9, *trans*-11 was the major positional isomer and 7,9 (*cis/trans* plus *trans/cis*) was the second most relevant peak. Noticeable increase ($P<0.05$) in the isomer 11,13 (*cis-trans/trans-cis* and *trans-trans*) CLA were also observed with SEL diet.

With respect to the content of *trans*-fatty acids, which because of their metabolic effects could be as prejudicial as saturated fatty acids with 12-16 carbons, Chilliard et al., [13] indicated that 5-15% of total quantity of C18:1 is *trans* configuration in goat milk. This was confirmed recently in sheep [34], goat [22], and cow [35] milks. *Trans*-C18:1 FA are produced during incomplete biohydrogenation of PUFA in the rumen and under most dietary conditions, *trans*-vaccenic acid (TVA, C18:1 *trans*11) is the predominant isomer in the three types of milk [36]. The total *trans*-C18:1 content increased 1.5-fold and 2.2-fold after incorporation of 0.5 kg and 0.7 kg SEL doses, respectively. A similar trend was also found in each *trans*-C18:1 isomer (Figure 2). Particularly, level of TVA significantly rose during the supplementation period, and its concentration in milk fat from goats fed a 0.7 kg SEL dose (2,92%) became 3-fold higher than with control diet (1.13 %).

Discussion

Saturated Fatty Acids

Recent advances in our understanding of the benefits of specific fatty acids in milk fat are of special significance due to the generally negative public perception that a food containing saturated fat is unlikely to be beneficial to human health [1]. The incorporation of both 0.5 kg or 0.7 kg of SEL to the goats' diet caused changes in milk fat composition containing a lower proportion of saturated fatty acids (SFA) than control (P<0,05) (Table 5). Largely the decrease in SFA level is caused by the lower concentrations of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) fatty acids. This result is particularly interesting since C14:0 and C16:0 are considered to be cholesterol-raising and are associated with the increased incidence of arteriosclerosis and coronary heart disease [37,38]. Moreover the concentration of SFA with a positive or neutral effect on human health (short fatty acids C4-C10 and estearic acid C18:0) remained almost constant (~29%). Short fatty acids (~ 19% in this study) are especially important because they are the responsible to the high level of medium chain triglycerides (MCT) in goat milk. Goat milk exceeds other milks in MCT content, which are of special interest from a therapeutic point of view, because their particular metabolism, and hence their application for treating the various gastro-intestinal disorders and diseases, besides its value in alleviating cow milk allergies [15,21].

PUFAs

These fatty acids have been associated with the decrease in the risk of heart disease and, at the same time, have been lacking in significant proportion in milk fat. To discuss the importance of the introduced changes on the milk fat composition of goat milk from a human health point of view, it is necessary to take into account the effects of some specific PUFAs.

Total CLA and CLA isomer distribution

Among animal feeding strategies for CLA enrichment of milk, those diets with linseed supplements have proved to be effective [17,18]. According to the data shown in Table 5, dietary linseed supplementation could be a valuable means of increasing CLA in goats' milk. Although no changes in CLA levels were detected in milk fat from 0.3 kg SEL-fed goats, we found that at higher supplement doses (0.5 kg or 0.7 kg) an increase in total CLA content was attained (P<0,05). Thus, dietary addition of 0.7 kg of SEL raised the level of total CLA from 0.78% in milk from basal diet-fed goats to 1.78% in milk from SEL-fed goats.

Table 6 shows the content as well as distribution of the different CLA isomers determined by Ag^{+}-HPLC. As can be seen C18:2 *cis*-9, *trans*-11 was the major positional isomer (> 90% of the total CLA). RA abundance would be consistent with the relative importance of the endogenous synthesis of CLA *cis*-9, *trans*11 from TVA by Δ-9 desaturase. The second most prevalent positional isomer (from a quantitative point of view) was CLA *cis*-7, *trans*-9.

Both RA and *cis*-7, *trans*-9 levels were positively affected (P<0.05) by the incorporation of SEL into the diet. Thus, RA contents were elevated from 7.22 to 16.36 mg/g CLA in 0.5 kg SEL-fed goats and from 4.66 to 12.58 mg/g CLA in 0.7 kg SEL-fed goats. Similarly, *cis*-7, *trans*-9 levels also showed sharp enhancements with SEL diet (Table 5). The most remarkable

increase (P<0.05) correspond to the isomer 11,13 (*cis-trans/trans-cis* and *trans-trans*). Similar results were also reported by Sanz-Sampelayo et al. [15].

Only limited data are available on the effects of diet on the distribution of individual CLA isomers, but it seems that dietary supplements high in α-linolenic acid may increase the relative proportion of 11,13 and 12,14 positional isomers [39,40]. Kraft et al., [41] hypothesized that the CLA isomer 18:2 *trans*-11, *cis* 13 and *trans*-11, *trans*-13 are formed in large quantities as a result of grazing mountain pasture, which is rich in α-linolenic acid. The pathway for the hydrogenation of this acid in the rumen involves an initial isomerization in which the double bond at the carbon-12 position is transferred to the carbon-11 position to form 18:3 *cis*-9, *trans*-11, *cis*-15, which is then reduced at both *cis* bonds to produce TVA through *trans*-11 *cis*-15 18:2 as intermediate [42,43]. Kraft et al., [41] also argued that the *trans*-11 double bond was the most stable *trans*-bond found among the 18:1 isomers and among the CLA isomers in ruminal fermentation. They proposed the existence of three different CLA isomers having a *trans*-11 double bond in milk: *cis*-9, *trans*-11 from bacterial synthesis in the rumen (via linoleic acid) plus mammary gland desaturation, and *trans*-11, *trans*-13 and *trans*-11, *cis*-13, both of bacterial origin in the rumen from α-linolenic acid as indirect precursor.

Long-Chain FA (Omega 6 and 3)

Linoleic and linolenic acids are interesting for human health, as an imbalance in the ratio of these two FA could increase the risk of cardiovascular diseases and some cancers (Williams, 2000; Simopoulus, 2004). In other supplementation trials, proportions of linoleic and linolenic acids in milk fat were not altered in goats fed canola oil [44] or oleic and sunflower oil [45]

but they increased slightly in goats fed unprotected canola seeds [46]. In this work the proportion of total n-3 fatty acids increased significantly (4-fold) in goats fed 0.7 kg SEL diet ($P<0.05$). Considering the present-day efforts to obtain a diet with lower ratio of ω-6/ω-3 fatty acids for the prevention and management of chronic diseases, the increase in 18:3 *cis*-9, *trans*-11, *cis*-15 and the slight decrease in C18:2 *cis*-9, *cis*-12 in milk from goats fed the SEL diet is interesting for human health. Thus, ω-6/ω-3 ratios of 3.95 and 2.57 were found for milk samples of goats that received 0.5 kg and 0.7 kg SEL respectively. Both values were below 5, which have been reported as the minimum acceptable value recommended for the ω-6/ω-3 ratio [47]

Trans-C18:1 profile

In our study, concentration of TVA after incorporation of SEL into the diet (at 0.5 and 0.7 kg/animal/day) was markedly higher than in control diet. Contrarily to data reporting alterations in the C18:1 *trans* profile in cows [35,48] attributed to modification in fermentation pattern and bacterial populations, TVA remained the most important isomer (Figure 2) and C18:1 *trans*-10 represented always less than (~30% at dose 0.5 y 29% at dose 0.7) 33% of TVA. Similar results were reported in goats fed canola seeds [32,49]. The discrepancy between these studies and our data may be due to the use of oilseeds rather than free oils, as less efficient ruminal FA biohydrogenation has been suggested when oil is free rather than a part of the seed [22].

Chilliard et al., [13] found a strong linear correlation between milk rumenic acid (CLA *cis*-9, *trans* 11) and C18:1 *trans*-11 percentages under a variety of diets in goats. The RA/TVA ratio represents an index that serves as a proxy for Δ^9 desaturase activity. In our study, goats fed 0.7 Kg SEL had a lower desaturase index that control group (0.41 vs 0.46). Andrade and

Schmidely, 2006, suggested that this reduction could be explained by an inhibition of Δ^9 desaturase activity caused by a higher availability of TVA and/or other intermediates of FA biohydrogenation.

Health promoting index (HPI)

Ulbritch and Southgate [50] proposed an atherogenic index (AI) for lipids as a dietary risk indicator for cardiovascular disease. The AI is the sum of the proportion in the fat of considered atherogenic or hyperlipidaemic fatty acids as lauric (C12:0) myristic (C14:0) and palmitic (C16:0) acids, divided by the proportion of total unsaturated fatty acids, i.e. monounsaturated (MUFA) plus polyunsaturated fatty acids (PUFA) considered anti-atherogenic and anti-thrombogenic fatty acids. More recently Chen et al., [26] used the inverse AI index to propos the health-promoting index (HPI) as an indicator for the prevention of human cardiovascular diseases of the lipids present in a food. The values of HPI for control diets were in accordance with those reported as average of milk products (around 0.44). The inclusion of SEL into the goats' diet resulted in a milk fat showing a higher HPI (0.41 for the control diet and 0.58 for supplemented diet) ($P<0.05$).

In order to compare the HPI index of dairy products with other food lipids, we propose that the sum of the proportion of the short chain fatty acids (SCFA) (C4-C10) (mainly present in milk fat) should be join the MUFA and PUFA as their established positive effect on human health.

$$HPI = [\Sigma SCFA + \Sigma MUFA + \Sigma PUFA] / (C12:0 + 4 \times C14:0 + C16:0)$$

This modification of the equation will result in a high HPI value for milk fat, closed to the values reported for considered healthy oils. Nevertheless, in this study the total proportion of

short fatty acids was not altered significantly ($P<0.05$) in goat's fed with SEL diet as we have described before (Table 4).

Conclusions

The SEL diet employed (particularly at dose of 0.7 kg/animal/day) allows us to improve the nutritional quality of the goats' milk, since increases the levels of PUFA especially CLA (RA and other minor isomers), TVA and C18:3 *cis*-9, *cis*-12, *cis*-15, and reducing simultaneously SFA content.

These findings suggest that the regime of feed supplementation employed has a great potential in changing goat milk fat composition towards dairy foods with FA composition more beneficial and could be used as an integral component of an overall strategy to improve the nutrition and health of the consumer.

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Figure Captions

Figure 1.

Temporal pattern development of total CLA concentration measured in milk fat from goats fed a supplement enriched in linseed (SEL) . During the treatment period (broken lines) three doses of SEL (0.3 –0.5 –0.7 kg/animal/day) were incorporated into the diet of herds A, B and C, respectively . Control diet (○) and Supplemented diet (●).

Figure 2.

Profile *trans* C18:1 fatty acids using SEL doses of 0.5 kg/animal/day (A) and 0.7 kg/animal/day (B). Control diet (■) and Supplemented diet (□). Mean values during the supplementation period.

Table 1. Ingredient composition of the control and supplemented diets used in the study

	(% of dry matter)	
Ingredient	**Control**	**SEL**
Dehydrated alfalfa	45	45
Corn	15	15
Beet pulp	10	10
Cotton seed	13	13
Control supplement	17	--
SEL supplement	--	17

Table 2. Chemical (%) and fatty acid composition (% of total fat) of the control supplement and the supplement enriched in linseed (SEL) used in studied diets at different levels (0.3, 0.5 and 0.7 kg/day/animal of SEL)

	Diets			
Component	**Control**	**SEL (0.3)**	**SEL (0.5)**	**SEL (0.7)**
Moisture	13.20	12.79	12.62	12.25
Protein	20.00	20.00	20.00	20.00
Fat	5.71	6.82	7.27	8.25
Ash	7.73	7.74	7.74	7.75
Calcium	1.00	1.00	1.00	1.00
Phosphorus	0.53	0.53	0.53	0.54
Fatty acid				
Palmitic acid	0.41	0.51	0.54	0.59
Stearic acid	0.05	0.13	0.15	0.20
Oleic acid	0.97	1.35	1.47	1.69
Linoleic acid	0.96	1.57	1.76	2.11
α-Linolenic acid	0.15	1.63	2.11	2.95
Total	**2.54**	**5.18**	**6.04**	**7.55**

Table 4. Compositional analysis and lipid profile of milk fat from fourteen goat herds. Two milk samples were collected weekly from each herd during 3 weeks. Values shown are means of 6 analyses (n=6).

Herds	Compositional Analysis				Lipid Profile			
	Fat (%)	Protein (%)	Lactose (%)	T.S. (%)	ΣSFA	ΣMUFA	ΣPUFA	CLA (%)
H 1	5.2 ± 0.61	3.6 ± 0.28	4.7 ± 0.13	14.0 ± 0.75	65.1 ± 0.64	23.9 ± 0.31	4.8 ± 0.16	1.2 ± 0.13
H 2	4.9 ± 0.34	3.7 ± 0.15	4.8 ± 0.04	13.9 ± 0.35	67.7 ± 1.29	20.7 ± 1.43	5.5 ± 0.23	1.2 ± 0.08
H 3	4.9 ± 0.41	3.6 ± 0.39	4.8 ± 0.15	13.9 ± 0.24	67.8 ± 2.83	22.1 ± 0.66	4.7 ± 1.03	1.1 ± 0.43
H 4	5.0 ± 0.24	3.5 ± 0.05	4.8 ± 0.06	13.9 ± 0.22	66.1 ± 0.77	23.3 ± 0.93	5.0 ± 0.13	1.0 ± 0.08
H 5	6.0 ± 0.30	4.0 ± 0.23	4.5 ± 0.16	15.0 ± 0.34	62.3 ± 1.71	29.3 ± 1.71	5.1 ± 0.12	0.9 ± 0.12
H 6	5.3 ± 0.39	3.7 ± 0.06	4.8 ± 0.03	14.2 ± 0.41	66.3 ± 0.97	25.5 ± 0.95	4.7 ± 0.10	0.6 ± 0.06
H 7	4.9 ± 0.28	3.8 ± 1.00	4.8 ± 0.06	14.1 ± 1.43	71.6 ± 0.70	21.6 ± 0.60	3.6 ± 0.05	0.6 ± 0.01
H 8	4.7 ± 0.41	3.7 ± 0.20	4.7 ± 0.12	13.7 ± 0.39	70.7 ± 0.83	22.9 ± 0.68	3.5 ± 0.21	0.6 ± 0.12
H 9	4.6 ± 0.17	3.8 ± 0.07	4.7 ± 0.05	13.6 ± 0.17	68.2 ± 3.59	25.8 ± 2.43	3.1 ± 0.52	0.6 ± 0.26
H 10	5.0 ± 0.25	3.5 ± 0.20	4.8 ± 0.09	13.9 ± 0.22	67.7 ± 5.15	25.5 ± 5.26	4.1 ± 0.21	0.4 ± 0.09
H 11	6.0 ± 0.52	3.4 ± 0.08	4.8 ± 0.05	14.8 ± 0.55	68.0 ± 0.81	26.7 ± 0.75	3.2 ± 0.10	0.4 ± 0.02
H 12	4.6 ± 0.41	3.8 ± 0.21	4.8 ± 0.08	13.6 ± 0.35	69.5 ± 1.09	25.1 ± 0.95	3.0 ± 0.12	0.4 ± 0.05
H 13	4.8 ± 0.38	3.7 ± 0.16	4.8 ± 0.10	13.8 ± 0.36	65.8 ± 0.96	27.5 ± 1.16	4.7 ± 0.13	0.4 ± 0.05
H 14	4.8 ± 0.12	3.5 ± 0.04	4.8 ± 0.06	13.6 ± 0.14	69.7 ± 1.00	25.4 ± 1.20	2.9 ± 0.11	0.3 ± 0.06
Mean	5.0 ± 0.35	3.7 ± 0.22	4.8 ± 0.08	14.0 ± 0.42	67.6 ± 2.41	24.7 ± 2.38	4.1 ± 0.90	0.7 ± 0.33

Table 3.

Milk composition in goats fed 3 levels of concentrate supplements for 30 days. Herds A, B, and C were supplemented with 0,3, 0,5 and 0,7 kg SEL per animal per day respectively. Supplemented diet (SD) and control diet (CD).

Item	Time (days)	Treatment					
		A		B		C	
		CD	SD	CD	SD	CD	SD
Fat (%)	0	5.46 ± 0.007	4.97 ± 0.170	6.00 ± 0.007	6.10 ± 0.255	4.73 ± 0.071	4.60 ± 0.151
	15	5.50 ± 0.127	5.65 ± 0.001	6.23 ± 0.205	6.13 ± 0.085	5.26 ± 0.191	5.01 ± 0.156
	30	5.54 ± 0.197	6.07 ± 0.575	6.86 ± 0.015	6.34 ± 0.340	5.27 ± 0.035	4.93 ± 0.148
Protein (%)	0	3.38 ± 0.150	3.49 ± 0.122	3.82 ± 0.021	3.67 ± 0.014	3.62 ± 0.049	3.55 ± 0.092
	15	3.36 ± 0.035	3.71 ± 0.028	3.87 ± 0.150	3.75 ± 0.064	3.68 ± 0.075	3.77 ± 0.050
	30	3.45 ± 0.053	3.86 ± 0.211	4.07 ± 0.053	3.87 ± 0.015	3.67 ± 0.014	3.88 ± 0.001
Lactose (%)	0	4.51 ± 0.010	4.49 ± 0.001	4.59 ± 0.015	4.61 ± 0.006	4.52 ± 0.212	4.49 ± 0.255
	15	4.88 ± 0.021	4.78 ± 0.021	4.47 ± 0.375	4.58 ± 0.085	4.72 ± 0.038	4.70 ± 0.050
	30	4.96 ± 0.021	4.93 ± 0.007	4.59 ± 0.006	4.53 ± 0.021	4.73 ± 0.001	4.78 ± 0.014
E.T.S. (%)	0	13.79 ± 0.077	13.83 ± 0.192	15.13 ± 0.064	15.12 ± 0.233	13.62 ± 0.113	13.85 ± 0.905
	15	14.39 ± 0.191	14.74 ± 0.014	15.54 ± 0.198	15.37 ± 0.042	14.27 ± 0.240	14.08 ± 0.236
	30	14.65 ± 0.244	15.63 ± 0.670	16.14 ± 0.426	15.95 ± 0.071	14.44 ± 0.042	14.26 ± 0.177

Table 4.

Main fatty acid composition of milk fat from goats after 30 days fed a control diet (CD) or supplemented diet (SD) Data expressed as percentage of total fatty acids (% FA). Herds A, B, and C were supplemented with 0.3, 0.5 and 0.7 kg SEL per animal per day, respectively.

Compound	A		B		C	
	CD	SD	CD	SD	CD	SD
C4:0	3.04 ± 0.049*	2.84 ± 0.071a	3.31 ± 0.187	3.47 ± 0.14b	3.30 ± 0.509	3.09 ± 0,427ab
C6:0	3.07 ± 0.033*	2.89 ± 0.035a	3.12 ± 0.172	3.16 ± 0.213a	3.17 ± 0.383	3.03 ± 0,207a
C8:0	3.45 ± 0.110	3.27 ± 0.136a	3.23 ± 0.186	3.19 ± 0.254a	3.38 ± 0.266	3.27 ± 0,084a
C10:0	10.43 ± 0.351	10.33 ± 0.257a	10.48 ± 0.450*	9.71 ± 0.608a	10.93 ± 0.519	10.15 ± 0,216a
C10:1	0.20 ± 0.012	0.22 ± 0.025a	0.28 ± 0.021	0.26 ± 0.034a	0.21 ± 0.105	0.28 ± 0,009a
C12:0	4.02 ± 0.124	4.28 ± 0.286a	4.73 ± 0.124*	3.96 ± 0.161a	4.62 ± 0.095*	4.27 ± 0,066a
iso C13: 0	0.02 ± 0.001*	0.04 ± 0.007a	0.06 ± 0.008	0.05 ± 0.005a	0.06 ± 0.010	0.07 ± 0,021a
ante-iso C13: 0	0.07 ± 0.005	0.10 ± 0.016a	0.14 ± 0.020	0.11 ± 0.009a	0.11 ± 0.002*	0.12 ± 0.003a
iso C14: 0	0.05 ± 0.001	0.05 ± 0.002a	0.05 ± 0.001*	0.04 ± 0.002b	0.05 ± 0.001	0.05 ± 0.002a
C14:0	8.09 ± 0.123	8.25 ± 0.214a	8.62 ± 0.025*	7.62 ± 0.228b	9.03 ± 0.034*	8.20 ± 0.172a
iso C15	0.12 ± 0.002	0.12 ± 0.005a	0.13 ± 0.002*	0.10 ± 0.001b	0.12 ± 0.003*	0.11 ± 0.003a
ante-iso C15	0.10 ± 0.004	0.14 ± 0.022a	0.20 ± 0.013	0.19 ± 0.012b	0.15 ± 0.005*	0.17 ± 0.005ab
C14:1	0.26 ± 0.060*	0.28 ± 0.059ab	0.34 ± 0.014*	0.27 ± 0.001a	0.27 ± 0.105	0.35 ± 0.003b
C15:0	0.55 ± 0.012	0.56 ± 0.015a	0.55 ± 0.008	0.48 ± 0.035b	0.57 ± 0.008*	0.60 ± 0.006a
iso C16	0.16 ± 0.008	0.15 ± 0.009a	0.15 ± 0.001*	0.11 ± 0.005b	0.16 ± 0.003	0.15 ± 0.003a
C16:0	23.26 ± 0.512	23.91 ± 1.019a	24.78 ± 0.441*	23.8 ± 0.532a	25.76 ± 0.483*	21.09 ± 0.361b
iso C17	0.38 ± 0.007	0.29 ± 0.056a	0.23 ± 0.011*	0.4 ± 0.025a	0.19 ± 0.001*	0.37 ± 0.001a
ante-iso C17	0.32 ± 0.010	0.31 ± 0.003a	0.28 ± 0.006	0.24 ± 0.029a	0.33 ± 0.013	0.31 ± 0.008a
C16:1	0.73 ± 0.005	0.85 ± 0.078a	1.03 ± 0.010	1.02 ± 0.064b	0.89 ± 0.031*	0.82 ± 0.022a
C17	0.40 ± 0.009*	0.38 ± 0.009a	0.32 ± 0.004*	0.24 ± 0.002b	0.26 ± 0.201	0.31 ± 0.006c
C17:1	0.19 ± 0.002	0.2 ± 0.007a	0.31 ± 0.041*	0.16 ± 0.001b	0.22 ± 0.016*	0.17 ± 0.005b
C18	10.59 ± 0.437	10.05 ± 1.019a	7.96 ± 0.204*	7.05 ± 0.217b	8.42 ± 0.498	8.70 ± 0.378ab
C18:1 *trans* (9+10+11)	4.41 ± 0.361*	3.52 ± 0.342a	2.95 ± 0.303*	6.02 ± 0.374b	2.18 ± 0.206*	4.30 ± 0.363a
C18:1	17.60 ± 0.845	18.62 ± 0.317a	19.61 ± 0.814	18.68 ± 0.504ab	18.63 ± 0.235*	19.82 ± 0.487b
C18:1 *trans*-15+*trans*-*11*	0.22 ± 0.003*	0.20 ± 0.001a	0.21 ± 0.001	0.25 ± 0.016ab	0.27 ± 0.008*	0.22 ± 0.004b
C18:1 *cis*-12	0.54 ± 0.008*	0.46 ± 0.008a	0.37 ± 0.015	0.37 ± 0.012b	0.21 ± 0.005*	0.36 ± 0.004b
C18:1 *cis*-13	0.62 ± 0.009	0.62 ± 0.056a	0.49 ± 0.022*	0.64 ± 0.040a	0.37 ± 0.023*	0.71 ± 0.007a
C18:1 *trans*-16+*cis*-14	0.05 ± 0.009	0.11 ± 0.055a	0.08 ± 0.008*	0.30 ± 0.038b	0.04 ± 0.003*	0.29 ± 0.002ab
C18:2 *trans*-11,*cis*-15	0.18 ± 0.092	0.14 ± 0.049a	0.11 ± 0.017*	0.82 ± 0.081b	0.07 ± 0.006*	0.66 ± 0.012c
C18:2 *cis*-9, *cis*-12	3.63 ± 0.114*	3.18 ± 0.173a	2.67 ± 0.064	2.55 ± 0.098b	2.67 ± 0.131	2,59 ± 0.075b
C20	0.14 ± 0.001	0.15 ± 0.005a	0.16 ± 0.005*	0.13 ± 0.003b	0.26 ± 0.032*	0.14 ±0.001ab
C18:3	0.34 ± 0.045	0.49 ± 0.191a	0.27 ± 0.016*	0.63 ± 0.047a	0.22 ± 0.004*	0.98 ± 0.069b
CLA	1.04 ± 0.050	0.97 ± 0.056a	1.01 ± 0.035*	2.02 ± 0.141b	0.72 ± 0.059*	1.76 ± 0.066c
Σ SFAs	67.09 ± 0.76	66.96 ± 0.73a	67.37 ± 0.99*	62.84 ± 1.10b	69.77 ± 0.95*	62.94 ± 1.12b
Σ MUFAs	20.41 ± 0.77	21.57 ± 0.28a	22.72 ± 0.86	21.40 ± 0.58a	21.11 ± 0.21*	23.03 ± 0.48b
Σ PUFAs	5.20± 0.123	4.78 ± 0.196a	4.05 ± 0.121*	5.75 ± 0.639ab	3.69 ± 0.188*	5.99 ± 0.203b
HIP	0.50 ± 0.024	0.49 ± 0.028a	0.46 ± 0.015*	0.57 ± 0.033b	0.41 ± 0.004*	0.57 ± 0.013b

Data are expressed as means ± SD (n=3)

[a,b,c] For supplemented diet (SD), means within the same row with different letters indicate significant differences between SEL doses (P<0.05)

* For each SEL dose, means with asterisk indicate significant differences between CD and SD (P<0.05).

Table 6. Conjugated linoleic acid (CLA) isomers (mg / g CLA) determined by silver-ion HPLC.

Isomers	Dose 0.5			Dose 0.7		
	CD	**SD**	***P***	**CD**	**SD**	***P***
trans-12, *trans*-14	0,02	0,028	0,488	0,03	0,033	0,388
trans-11,*trans*-13	0,02	0,063	0,007	0,02	0,109	0,006
trans-10,*trans*-12	0,02	0,091	0,005	0,01	0,086	0,001
trans-9,*trans*-11	0,04	0,033	0,028	0,04	0,026	0,006
trans-8,*trans*-10	0,03	0,063	0,037	0,02	0,063	0,021
trans-7,*trans*-9	0,02	0,011	0,043	0,03	0,009	0,201
trans-6,*trans*-8	0,01	0,014	0,341	0,01	0,015	0,809
Σ trans/trans	*0,18*	*0,305*	0,026	*0,18*	*0,327*	0,019
cis,trans/*trans*,*cis* 12,14	0,03	0,047	0,040	0,01	0,051	0,032
trans-11,*cis*-13	0,01	0,100	0,022	0,01	0,196	0,004
RA	7,22	16,358	0,001	4,66	12,583	0,001
cis-7,*trans*-9	0,47	0,838	0,004	0,34	0,598	0,003
Σ cis/trans + trans/cis	*7,73*	*17,343*	0,001	*5,02*	*13,428*	0,001

t: trans double bond; **c:** cis double bond ; RA: C18:2 *cis*-9**,** *trans*-11; **CD :** control diet ; **SD :** supplemented diet. Level of significance, p<0.05.

Fig 1. Temporal pattern development of total CLA concentration measured in milk fat from goats fed a supplement enriched in linseed (SEL). During the treatment period (broken lines) three doses of SEL (0.3 –0.5 –0.7 Kg/animal/day) were incorporated into the diet of herds A, B and C, respectively.

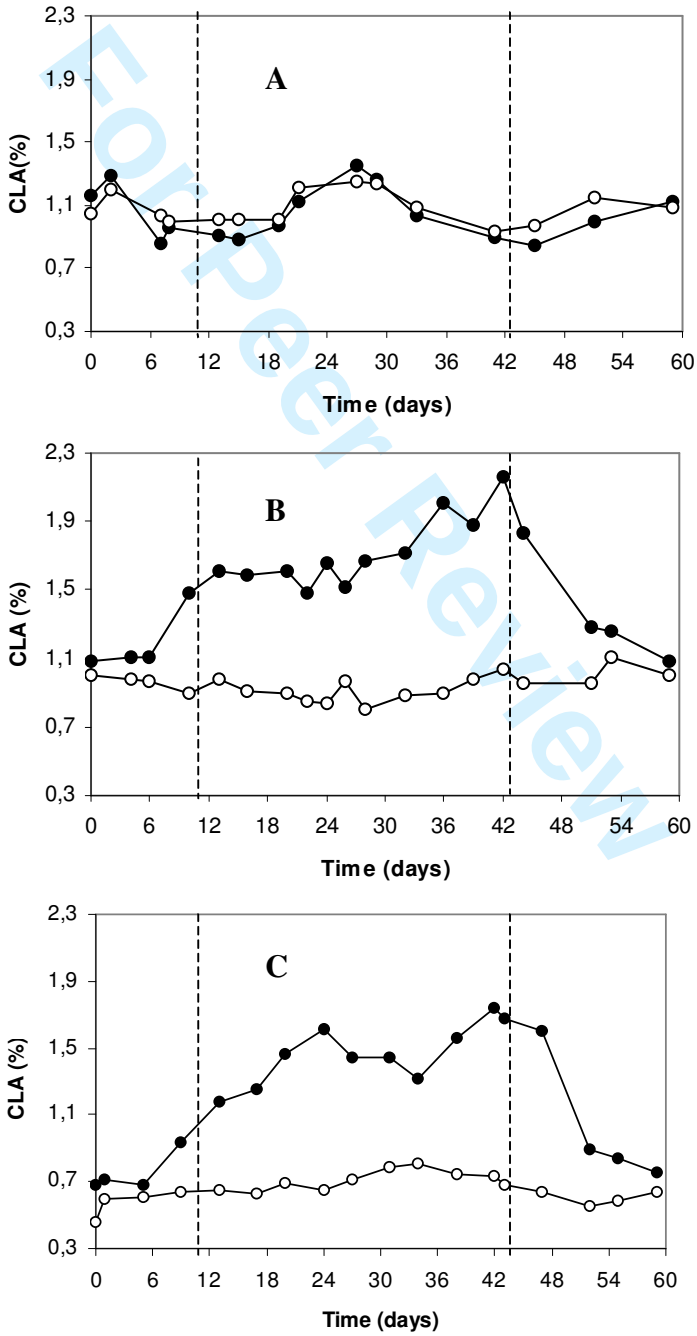
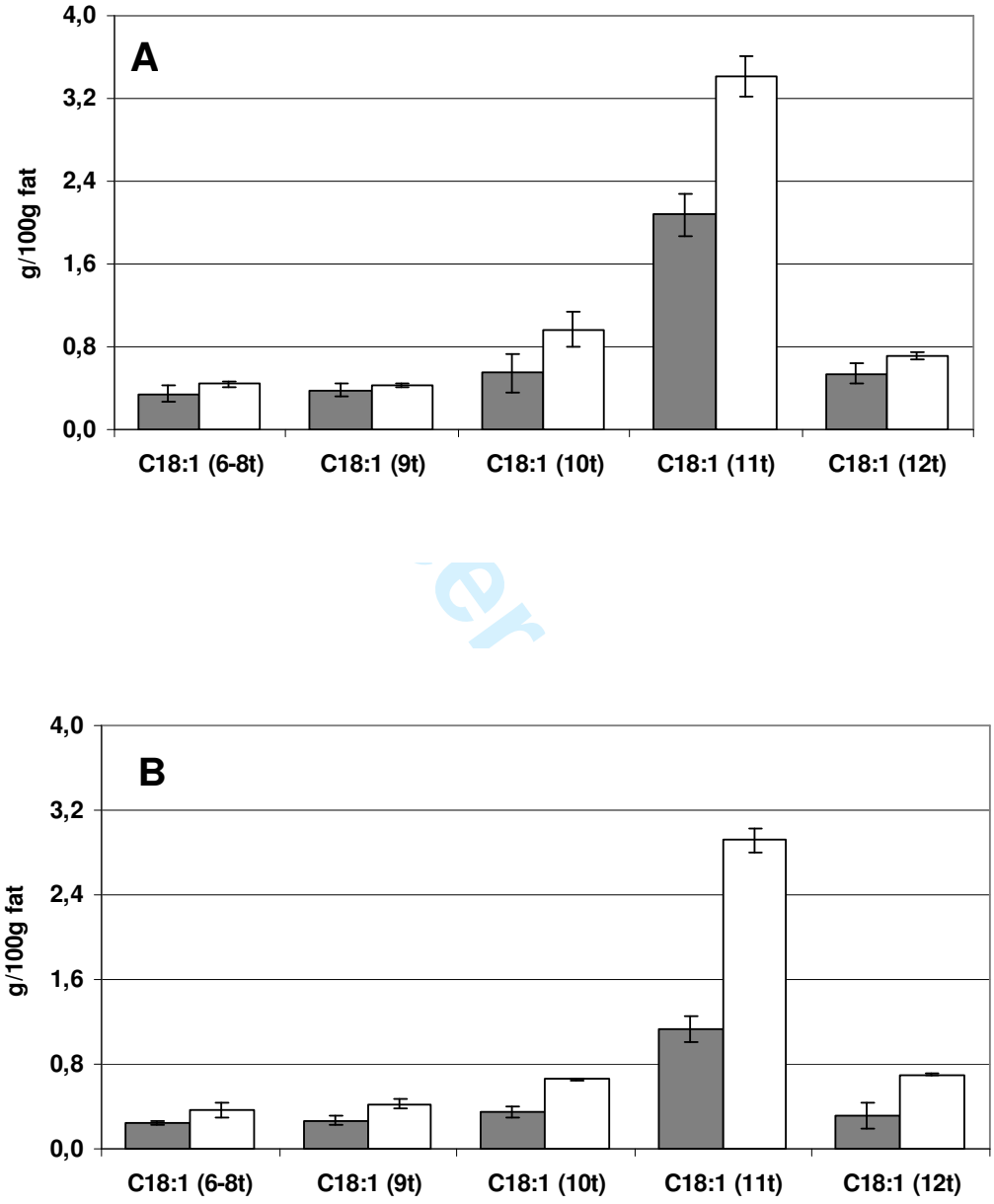


Fig 2. Profile *trans* C18:1 fatty acids using SEL doses of 0.5 kg/animal/day (A) and 0.7 kg/animal/day (B). Control diet (■) and Supplemented diet (□). Average Data over supplementation period.



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Abstract: Bifidobacterium and lactic acid bacteria (LAB), especially from the genera Lactobacillus and Lactococcus, are commonly used due to their potential probiotic characteristics, to produce fermented dairy products. Moreover, some strains of these microorganisms also have the ability to produce conjugated linoleic acid (CLA) from linoleic acid (LA), which has attracted much attention as a novel type of beneficial functional fermented milk.

In the present work twenty two probiotic bacteria of the genera Bifidobacterium, Lactobacillus and Lactococcus were tested for the production of CLA, using a UV screening method and HPLC techniques. It have been possible to quantify (40-50 μg CLA/mL) and identify the CLA isomers: C18:2 c9, t11 (60-65%), C18:2 t10, c12 (30-32%) and C18:2 t9,t11 and t10,t12 (2-5%) produced by 5 selected probiotic microorganisms incubated in skim milk with free linoleic acid as a substrate.

Dear Professor Gordon Birch,

In the present work “Quantitative and qualitative determination of CLA produced by Bifidobacterium and LAB by combining spectrophotometric and Ag+-HPLC techniques” it is reported the identification of potencial probiotic bifidobacterium and lactic acid bacteria able to produce conjugated linoleic acid isomers (CLA) in skim milk using linoleic acid (LA) or safflower oil as substrate by combining UV spectophotometry and Ag^{+}-HPLC. To that aim 22 bacteria were firstly screened to their ability to produce CLA isomers in growth media. Then 5 strains showing this characteristic were tested in skim milk to adjust the conditions of reaction and time of incubation. A final trial was preformed with the selected probiotic bacteria and the optimal conditions previously assayed for skim milk and the CLA isomers produced identified and quantified by Ag+-HPLC which allows separating the conjugated diene fatty acids selectively.

CLA have been reported to exert numerous beneficial biological effects leading to a high interest in their inclusion in foods. Due to milk and dairy products are rich sources these compounds as result of the biohidrogenation of polyunsaturated fatty acids in the rumen of the ruminants by microbial enzymes have been suggested that other bacteria may have this capability. *Lactobacillus*, *Bifidobacterium* and *Lactococcus*, are commonly used, due to their potential probiotic characteristics, to produce fermented dairy products. The identification of LAB able to produce CLA from a source of LA is of great importance since their use in the production of fermented dairy products will be of interest for human consumption as a probiotic dairy product with high CLA content.

CLA can be analyzed by chromatographic techniques that are time consuming. On the other hand, UV spectrophotometry gives a rapid measurement of the content of CLA in samples and combining with Ag^{+}-HPLC can be achieved the qualitative analysis, knowing the distribution of the conjugated linoleic acid isomers produced by these bacteria. The combining these techniques would permit not only the screening but the determination of the different CLA isomers. Not previous paper reporting these methodologies have been published.

Sincerely,

Javier Fontecha

Quantitative and qualitative determination of CLA produced by Bifidobacterium and LAB by combining spectrophotometric and Ag+-HPLC techniques.

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ABSTRACT

Bifidobacterium and lactic acid bacteria (LAB), especially from the genera *Lactobacillus* and *Lactococcus*, are commonly used due to their potential probiotic characteristics, to produce fermented dairy products. Moreover, some strains of these microorganisms also have the ability to produce conjugated linoleic acid (CLA) from linoleic acid (LA), which has attracted much attention as a novel type of beneficial functional fermented milk.

In the present work twenty two probiotic bacteria of the genera *Bifidobacterium*, *Lactobacillus* and *Lactococcus* were tested for the production of CLA, using a UV screening method and HPLC techniques. It have been possible to quantify (40-50 μg CLA/mL) and identify the CLA isomers: C18:2 c9, t11 (60-65%), C18:2 t10, c12 (30-32%) and C18:2 t9,t11 and t10,t12 (2-5%) produced by 5 selected probiotic microorganisms incubated in skim milk with free linoleic acid as a substrate.

1. Introduction

Conjugated linoleic acid (CLA) is a mixture of positional and geometric conjugated isomers of the essential fatty acid linoleic acid (LA) with conjugated double bonds at carbon positions from 6-8 to 13-15. CLA isomers (C18:2 c9,t11 and C18:2 t10,c12) confer a number of beneficial biological effects that have been identified in a range of animal models and include anti-carcinogenesis, immunomodulation, anti-atherosclerosis and reduction of whole body fat (Hur, Park & Joo, 2007; Lin, Lin & Lee, 1999; Park & Pariza, 2007; Tanaka, 2005).

These compounds occur naturally in a variety of foods, including ruminant products such as milk fat and meat which have been found to contain relatively large amounts of CLA. Dairy products from ruminants are very rich sources of CLA, among which 18:2 *cis*-9, *trans*-11, is the main isomer (Chin, Liu, Storkson, Ha & Pariza, 1992). The presence of these compounds in dairy products is partly due to the isomerization and biohydrogenation of linoleic and linolenic acids that take place in the rumen; these processes are performed by ruminal bacteria, such as *Butyrivibrio fibrisolvens* and *Megasphaera elsdenni* (Bauman & Griinari, 2003; Jouany, Lassalas, Doreau & Glasser, 2007; Sieber, Collomb, Aeschlimann, Jelen & Eyer, 2004). Such observation has raised the hypothesis that other microorganisms may also be able to produce CLA. This hypothesis and the fact that several fermented dairy products contain higher levels of CLA than non-fermented counterparts, created the possibility of producing fermented dairy products with high levels of CLA. Lactic acid bacteria (LAB), especially from the genera *Lactobacillus*, *Bifidobacterium* and *Lactococcus*, are commonly used, due to their potential probiotic characteristics, to produce fermented dairy products (Parvez, Malik, Kang & Kim, 2006; Saarela, Mogensen, Fondén, Mättö & Mattila-Sandholm,

2000). The identification of LAB able to produce CLA from a source of LA is of great importance since their use in the production of fermented dairy products will be of interest for human consumption as a probiotic dairy product with high CLA content.

Gas chromatography systems fitted with polar capillary columns and FID detectors are widely used in the fatty acid routine analysis (Jensen, 2002), however in the identification and quantification of minor compounds as well as when several isomers are presented, a combination of methodologies is needed. In analysis of CLA, GC has to be combined with Ag^{+}-HPLC, obtaining a full resolution of all the CLA isomers in the sample (Bondia-Pons, Molto-Puigmarti, Castellote & Lopez-Sabater, 2007; Sehat, Yurawecz, Roach, Mossoba, Kramer & Ku, 1998). Furthermore HPLC and GC are time consuming but due to conjugated double bonds can be detected using a 233 nm wavelength UV spectrophotometers are able to perform a simple and rapid measurement in the high CLA producer LAB screening assays (Barrett, Ross, Fitzgerald & Stanton, 2007).

The aim of the present research work is to select CLA-producing bacteria in skim milk from a pool of potential probiotic LAB, using a UV screening method to measure the CLA concentration followed by HPLC analytical techniques that are able to detect and identify the CLA isomers, toward their future application in the manufacture of fermented products.

2. Material and Methods

2.1 Analitical reagents

All reagents used in lab procedures were HPLC grade: hexane and isopropanol were obtained from Labscan (Dublin, Ireland), rumenic acid and linoleic acid from Sigma-Aldrich (St. Louis, MO, USA) and high CLA content oil (Tonalin®) from Cognis (Illertissen, Germany).

2.2 Bacterial strains

Twenty-two potentially probiotic strains were selected for this study; these included 16 strains of *Lactobacillus*, 5 strains of *Bifidobacterium* and 1 strain of *Lactococcus lactis* (table 1).

2.3 Screening for the bacteria producers of CLA

The first screening to select the higher CLA producer bacteria was performed using culture medium. The bacterial strains were activated overnight at 37 ºC in M17 broth (Oxoid, Hampshire, UK) (for *Lactococcus lactis*), MRS broth (Pronadisa, Madrid, Spain), supplemented with 0.05 % (w/v) of pure cystein (Sigma) (for the *Lactobacillus acidophilus* and the Bifidobacterium strains) incubated under anaerobic conditions and MRS broth for the remaining strains. Five percent (v/v) of these activated cultures were transferred to the appropriate culture medium (10ml) containing 1 % (w/v) Tween-80 (Scharlau, Sentmenat, Barcelona, Spain) and 1 mg/ml of linoleic acid (Sigma) and incubated for 24h under the same conditions as described above.

Only the strains that produce higher CLA concentration in the medium were also tested in skim milk. To this aim, the selected bacteria were activated again in an overnight culture under the conditions described above. Then 10 ml of skim milk (Scharlau, Sentmenat, Barcelona, Spain; 0.5 g fat/L) (10 % w/v) was inoculated with those activated cultures (5 % (v/v)) and incubated under the respective conditions for 24h. Afterward, 2 % (v/v) was transferred to 10 ml skim milk (10 % (w/v)) containing 1 % (w/v) tween 80 and 1 mg/ml linoleic acid or 1 mg/ml safflower oil and incubated at 37 ºC under the respective atmosphere conditions of each bacterium during 24h or 48 h respectively. Using two linoleic acid sources (from free fatty acids and from safflower oil) allowed the authors to know if substrate affect to the CLA production.

2.4 Lipid Extraction

Lipid isolation from culture media was carried out as a variation of the method described by Alonso et al. (2003). Briefly: 10 mL of culture media was centrifuged at 10000 rpm, 10 min, 4ºC. Three mL from the supernatant was added with 6 mL of isopropanol and vortex 1 minute, next addition of hexane (5 mL) followed by 1 minute vortexing. For a better separation of the organic and aqueous phases a new centrifugation step was arranged (2000 rpm, 5 min, 4º C).

2.5 Quantification of CLA production by UV spectroscopy

Total CLA determination was carried out at a wavelength of 234 nm in a Perkin-Elmer spectrophotometer (Lambda650 model, Beaconsfield, UK) with a scan program (190-350 nm). Measurements were obtained by triplicate from 2 mL of the lipid extract in hexane placed into quartz cuvettes.

The concentration of CLA produced by the analyzed LAB was determined by means of a standard curve prepared with a solution of CLA (C18:2 c9, t11) in hexane containing 0.746 mg/mL and diluted at different concentration points (0, 5, 10, 15, 20 and 30 µg/mL).

2.6 CLA isomers determination by Ag^{+}-HPLC.

CLA isomers profile produced by LAB in skim milk was determined by Ag^{+}-HPLC. Previously, 4mL of extract was evaporated, and methylated using sulphuric acid in mild conditions according to Aldai et al. (2005) to obtain the FAME extract.

Separation of CLA methyl esters was carried out using a HPLC system (Shimadzu Vp Series, Duisburg, F.R. Germany) equipped with UV detector operated at 233 nm. FAMEs were separated using three ChromSpher 5 Lipid analytical column (4.6 mm i.d. X 250 mm stainless steel; 5 µm particle size; Varian, Palo Alto, CA, USA). The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL/min. The flow was initiated 0.5 h prior to the sample injection (10µl).

161

162 *Statistical Analysis*

163

164 An orthogonal ANOVA analysis with two factors Var1 (five levels: *B. animalis* BLC,
165 *B. animalis* Bb12, *L. acidophilus* Lac1, *L. plantarum* and *Lactococcus lactis*) and Var2
166 (four levels: linoleic acid 24h, control 24h, safflower 48h and control 48h) was
167 conducted. The homogeneity of variances was tested by Cochran's test ($p=0.682$). A
168 significant interaction was found between Var1 and Var2 so Newman Keuls tests were
169 performed. All statistical analyses were performed using Statistica 6.1 (Statsoft).

170

3. Results

The standard curve of pure CLA solutions at different concentrations of rumenic acid (RA) in hexane (from 0 to 30 µg/mL) determined by UV absorption at 234 nm was arranged. The measurements were done in triplicate (Fig 1). The graph demonstrates that increasing the CLA concentration coincided and the linearity obtained was R^2 =0.993 to an absorbance of 2.2. Nevertheless, in order to confirm the results, a second calibration curve was arranged using Tonalin® (CLA-TG80 oil; 80% CLA) in hexane solution applying the RA regression curve equation and obtaining also a good linearity (R^2=0.997). This results agree with previous studies that demonstrated no differences among pure CLA standards and high CLA oils (Barrett *et al.*, 2007).

Therefore the CLA concentration in the lipid extract of the culture supernatant's can be calculated from the linear trend of the standard curve. The lipid extracts, obtained from the 22 bacteria assayed in this work, were screened spectrophotometrically at wavelength of 233nm for CLA production following growth in the presence of linoleic acid. With this approach, a total of 6 lipid extracts of the 22 bacteria assayed in this work were identified with the ability to transform LA into CLA (Table 1).

Subsequently each of the 6 CLA producing strains were afterwards assayed for their ability to substrate transformation (LA or Safflower oil) and optimum incubation time 24h and 48h (Table 2.). The CLA production data was calculated using the previous standard curve equation but taking into account that the recovery percentage of CLA in skim milk was of 82.24% (by using a blank of rumenic acid without the presence of LAB, data not shown). The higher levels of CLA production occurred for *L. acidophilus Lac1* and *B. animalis BLC* (41.6 and 36.3 µg/mL) at 24 h of incubation when safflower oil was added as a substrate to the milk media, although other strains increased the

196 bioconversion of linolenic acid to CLA production at 48h of incubation time.
197 Nevertheless, when free LA was used as substrate the higher rate of bioconversion of
198 CLA was obtained for *B. animalis Bb12* (21.6 µg/mL) at 24 h of incubation time.
199 Finally, a third trial of experiments was carried out in the optimum conditions in order
200 to test the proliferation and viability of producing a high CLA probiotic dairy product.
201 *Lactobacillus acidophilus* ki was excluded in the studies due to the very low CLA
202 production in skim milk. Therefore the 5 selected strains for a high CLA production
203 were grew by duplicate in skim milk with free LA added as substrate and incubated
204 during 24 hours. The same strains were incubated with safflower oil during 48 hours.
205 In what concerns to bacteriological growth, in the presence of both LA and Safflower
206 oil all the strains used were able to grow. The highest growth in the presence of LA was
207 achieved by *L. lactis* and the same occurred when safflower oil was added to skim milk
208 (figure 2a and 2b). Therefore, the presence of these subtracts did not have any influence
209 upon the bacteriological growth ($p > 0,05$) during the incubation time.
210 All of the CLA producing bacteria displayed homology values of bioconversion of LA
211 and all were found to be around 40-50 µg/mL when incubated with free LA during 24h
212 and around 20 µg/mL when incubated with safflower oil during 48h (with the exception
213 of *L. acidophilus Lac1* with only 4.7 µg/mL) (Table 3).
214
215 Due to that the spectrophotometric method applied does not distinguish between
216 isomers of CLA since this determination is based on the measurement of the conjugated
217 double bound in the fatty acid, chromatographic analysis by Ag^{+}-HPLC was carried out.
218 The chromatogram profile (Fig. 3) showed the presence of 4 major conjugated linoleic
219 moieties confirmed by the second derivative of the spectra (Banni, Day, Evans,
220 Corongiu & Lombardi, 1995) and by the injection of pure CLA standards. As expected,

the C18:2 c9, t11 (rumenic acid) was the predominant isomer generated with a 60-65% and also the C18:2 t10, c12 that accounted a 30-32%. The same fatty acids isomers but with *trans, trans* configurations (9,11 and 10,12) were also generated as a minor compounds (2-5%). Similar profile was obtained for all the 5 selected strains.

4. Discussion

In addition to the increased interest in the physiological effects conferred upon humans following CLA consumption, there has been concomitant interest in the isolation bacterial strains (*Bifidobacterium* and LAB, especially from the genera *Lactobacillus* and *Lactococcus*) with the ability to produce CLA in milk or dairy products (Alonso et al., 2003; Bisig, Eberhard, Collomb & Rehberger, 2007; Ogawa, Kishino, Ando, Sugimoto, Mihara & Shimizu, 2005; Sieber et al., 2004). Furthermore, the combination of UV spectrophotometric and chromatographic techniques turns possible a rapid identification from a pool of microorganisms, those able to produce a high quantity of CLA and at the same time quantify the amounts produced while GC and/or HPLC techniques allow the quantitative and qualitative analysis of the isomers (Barrett et al., 2007; Wang, Lv, Chu, Cui & Ren, 2007).

Previous works have reported the ability of Bifidobacteria and LAB to produce CLA in a growth media with LA as substrate at concentrations ranged from 3,5 to 350 µg/mL with *Bifidobacterium*, 60 to 1500 µg/mL with *Lactoccocus* and 20 to 4900 µg/mL with *Lactobacillus* (Ogawa et al., 2005; Sieber et al., 2004). In the present work when a screening to identify possible CLA producers was performed, the amounts registered in the positives cases where under the results obtained for other authors as showed above, except for *Bifidobacterium animalis Bb12* and *BLC*. The differences found among our results and those obtained by other research groups may be due to intrinsic

247 characteristics of the microorganisms and methodologies (amount of LA, time and
248 temperature of incubation) as well as the fact that LA would have antimicrobiological
249 characteristics (Nieman, 1954), and that the isomerization of CLA was proposed as a
250 detoxify mechanism that even may produce saturated fatty acids (Adamczak,
251 Bornscheuer & Bednarski, 2008).
252 When oils are used as LA sources, bacteria must have the ability to produce lipases and
253 esterases to release the fatty acid from the triacyglycerides and it is well established that
254 some strains are able to do it (Holland et al., 2005), what in fact represent an extra
255 reaction step. According to this, although our results showed a good CLA production
256 during incubation in skim milk with safflower during 24 hours (table 2), we chose for
257 the last experiment 48h as reaction time due to the CLA concentration seem to increases
258 in some of the selected strains. At contrary, CLA production was lower for those strains
259 than before (table 3). Decreases of CLA production due to oxidation reactions as well as
260 oxidative metabolism of the microorganisms have been reported (Ogawa, Matsumura,
261 Kishino, Omura & Shimizu, 2001; Wang et al., 2007). This effect may explain the
262 decrease of CLA production in skim milk using either LA or safflower as substrate
263 when the incubation time was longer than 24h.
264 Other authors (Xu, Boylston & Glatz, 2004) have reported no increments in the CLA
265 concentration when the assay was carried out in skim milk adding milk-fat or soya oil
266 (total fat content 1%), during 24h incubation when using *P. freudenreichii*, *L.*
267 *acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *E. faecium*, *P. acidilactici*, y *B.*
268 *bifidum*. However when hydrolyzed soya oil was added (total fat content 1%) CLA
269 production was 0.63-2.21 mg CLA/g fat. Puniya et al, (2008) found that *L. brevis*
270 isolated from rumen fluid produced 10 mg CLA/g fat in skim milk using sunflower oil
271 (0.25%) as a source of LA, while *L. lactis* with 1% sunflower oil was 9.22 mg CLA/g

fat. The former research work support the results presented in this study where concentrations of 2.72-3.44 mg CLA/g fat was obtained in skim milk after 24h using LA as substrate while lower concentrations (0.31-1.53 mg CLA/g fat) were produced when safflower was added

Several authors (Kishino, Ogawa, Ando, Omura & Shimizu, 2002; Ogawa et al., 2005) have proposed pathways to CLA formation by LAB using LA. The fatty acid is converted into 10-hidroxy-12-*trans*-octadecadienoic and 10-hidroxy-12-*cis*-octadecadienoic and finally towards C18:2 c9, t11 (RA) and C18:2 *trans* 9, *trans* 11. It is generally accepted that LAB can transform polyunsaturated fatty acids to hidroxy fatty acids (Kim, Park, Chung, Kim, Kim & Kyung, 2003). Our results show that the selected probiotic strains produce CLA from LA and it was transformed mainly in C18:2 *cis* 9, *trans* 11 followed for C18:2 *trans* 10, *cis* 12 and small amounts of *trans*, *trans* isomers (9,11 and 10,12). Ogawa et al. (2005) supported all this results when reviewed the CLA profile produced by *Bifidobacterium* and *Lactobacillus* strains when growing in the presence of LA. Other studies elsewhere (Coakley, Ross, Nordgren, Fitzgerald, Devery & Stanton, 2003) reported that *Bifidobacterium* species were able to isomerize LA to CLA, giving an isomer profile of C18:2 *cis*9 *trans* 11 and C18:2 9t, 11t while *Lactoccocus* and *Lactobacillus* did not showed this ability. According to these authors the presence reported of the C18:2 10t, 12t could be formed by the conversion of C18:2 10t, 12c.

In this assay, the isomer composition produced by the probiotic bateria studied is alike to those found in synthetic mixtures (Ma, Wierzbicki, Field & Clandinin, 1999) being a good alternative to avoid the chemical reagents if production was above the reported levels.

All the CLA producers strains studied in this work may be used in the dairy industry, namely in milk, in order to produce dairy products with increased CLA content, because subtracts that can be used for that showed no inhibition. Future studies should be performed in order to optimize the CLA production and the probiotic growth in the presence of LA rich milk as substrates.

5. Conclusion

In this work it have been developed a throughput procedures that combine spectrophotometic and Ag+-HPLC that are able to determine the CLA production capabilities of *Bifidobacterium* and lactic acid bacteria, using different sources of linoleic acid such as free or oil. The developed working conditions including the fatty acid substrate, time and temperature to perform these assays are critical and therefore a good alternative to the high interest of dairy industry to obtain probiotic fermented products containing high levels of CLA. Future investigations should be carrying along in order to improve and achieve the optimal production conditions at large scale.

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Tables

Table 1

CLA concentration (µg/mL) of the culture supernatant's obtained after incubation of the strains in MRS medium with free LA (1mg/mL) for 24 h and calculated spectrophotometrically at wavelength of 233nm from the linear trend of the standard curve.

Strain	µg CLA /mL MRS[a]
L. brevis	-
***L. acidophilus* Lac 1**	-
***B. animalis* Bb12**	7.12 (0.054)
***B. animalis* BLC**	6.93 (0.078)
L. casei 1	-
***L. paracasei* ssp. *paracasei* LCS**	-
***L. acidophilus* Ki**	8.57 (0.102)
***B. animalis* Bo**	-
L. casei 2	-
L. rhamnosus 1	-
***L. casei* 01**	-
***L. acidophilus* La5**	-
***B. animalis* Bb12**	-
L. casei 3	-
L. rhamnosus 2	-
L. plantarum 1	-
***L. acidophilus* Lac1**	3.89 (0.098)
***B. animalis* BLC**	-
L. plantarum 2	6.77 (0.025)
***L. casei* LCS**	-
***L. acidophilus* ATCC 4356**	-
L. lactis	6.73 (0.045)

[a]Results expressed as mean values of triplicate determination (coefficient of variation, CV).

Table 2

CLA concentration (μg/mL) of the culture supernatant's obtained after incubation of the selected strains in MRS medium with free LA (1 mg/mL) and safflower oil (1mg/mL) for 24 and 48 h and calculated spectrophotometrically at wavelength of 233nm from the linear trend of the standard curve.

	μg CLA/ mL skim milk							
Strain	**Free Linoleic Acid**				**Safflower oil**			
	24h		**48h**		**24h**		**48h**	
	Mean	**CV**	**Mean**	**CV**	**Mean**	**CV**	**Mean**	**CV**
***B. animalis* Bb12**	21.57	0,089	10.17	0,075	16.72	0,036	26.06	0,089
***L. acidophilus* Lac1**	15.29	0,067	0.89	0,100	41.62	0,058	16.27	0,067
***B. animalis* BLC**	20.46	0,100	13.86	0,102	36.33	0,082	24.41	0,100
L. lactis	18.94	0,043	13.35	0,057	24.03	0,055	32.80	0,043
***L. acidophilus* Ki**	2.31	0,067	0.89	0,076	7.08	0,105	8.01	0,067
L. plantarum	11.44	0,086	17.54	0,105	10.30	0,046	18.82	0,086

Results expressed as mean values of triplicate determination (coefficient of variation, CV).

Table 3

CLA concentration (μg/mL) of the culture supernatant's obtained after incubation of the selected strains in skim milk medium with free LA (1mg/ mL) during 24h and safflower oil (1mg/mL) for 48h and calculated spectrophotometrically at wavelength of 233nm from the linear trend of the standard curve.

	μg CLA/ mL Skim Milk			
Strain	**Free Linoleic Acid**		**Safflower oil**	
	Mean	**CV**	**Mean**	**CV**
L. lactis	45.51	0,062	23.05	0,087
B. animalis BLC	48.25	0,054	18.41	0,089
B. animalis Bb12	42.21	0,106	22.29	0,091
L. acidophilus Lac1	40.94	0,070	4.68	0,058
L. plantarum	51.68	0,052	21.53	0,102

Results expressed as mean values of triplicate determination (coefficient of variation, CV).

Figure(s)

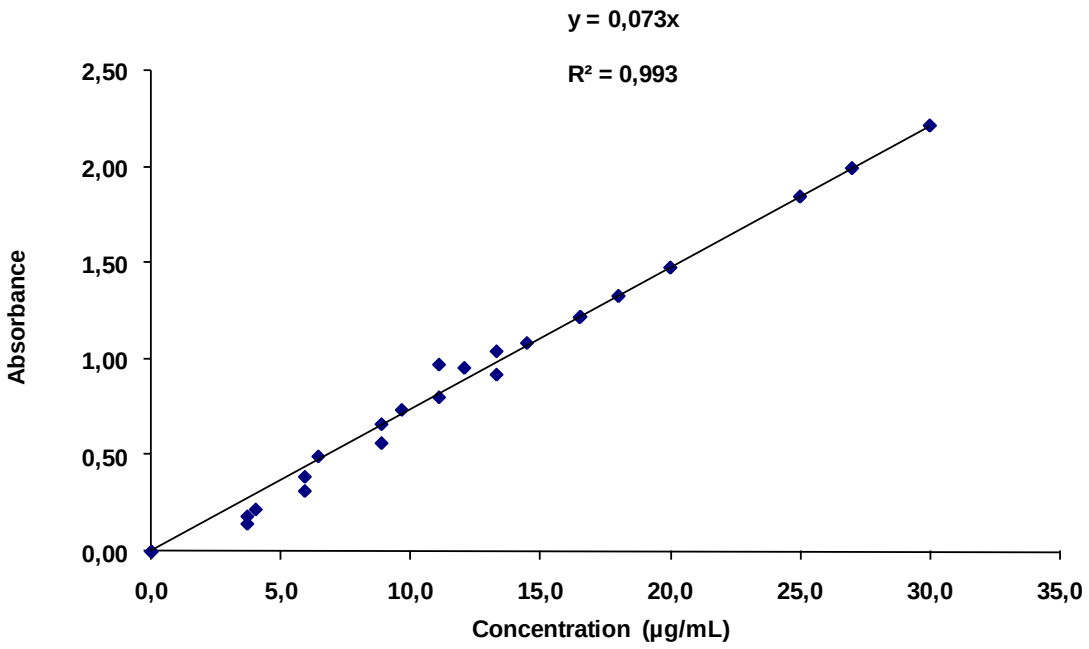

Fig. 1. Standard curve of the absorbance at wavelength of 233nm versus CLA standard (C18:2 c9,t11) at different concentrations.

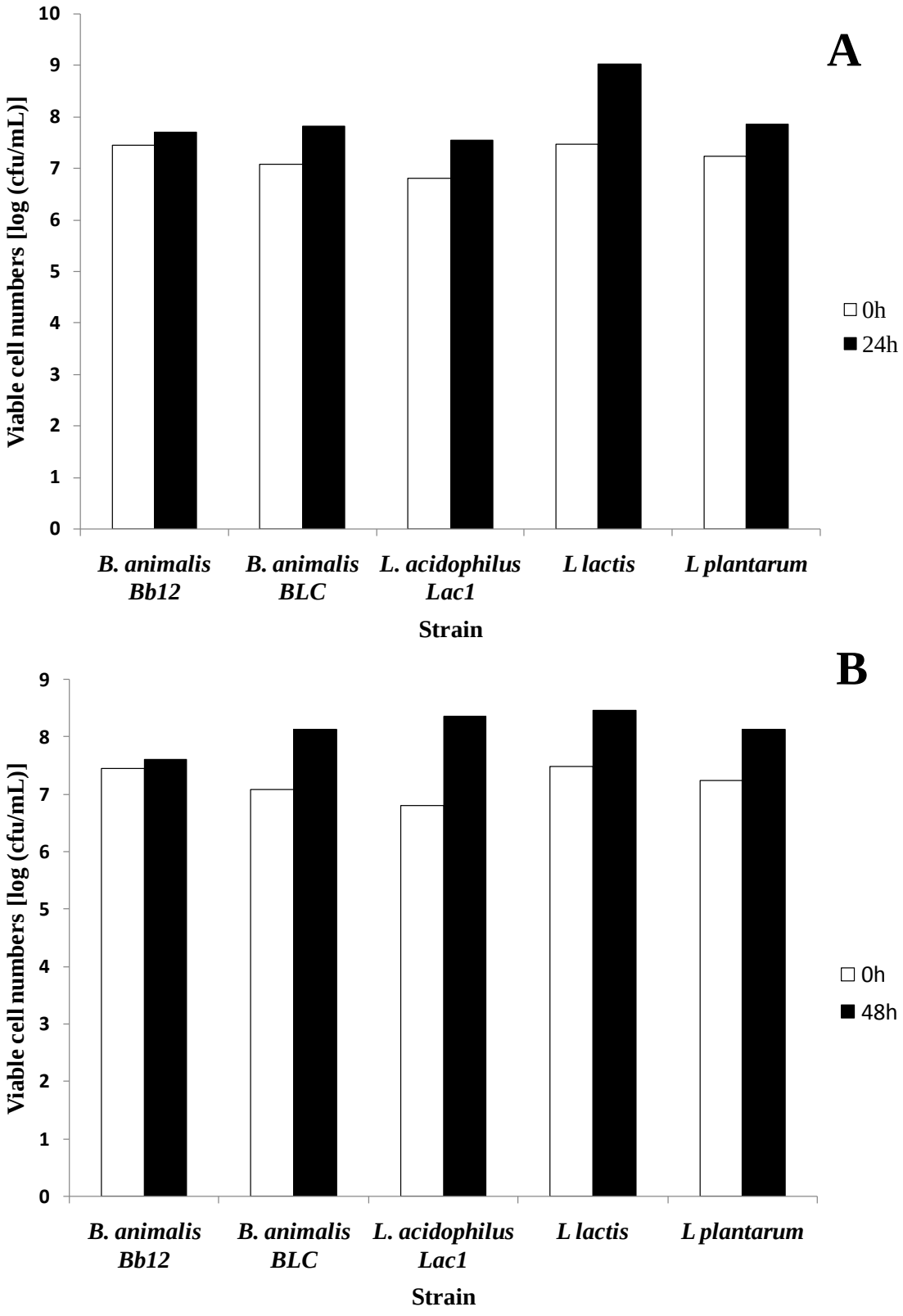

Fig. 2. Viable cell numbers [log (cfu/mL)] of selected strains cultured in skim milk with free linoleic acid (A) or safflower oil (B) added, and incubated 24 and 48 h respectively.

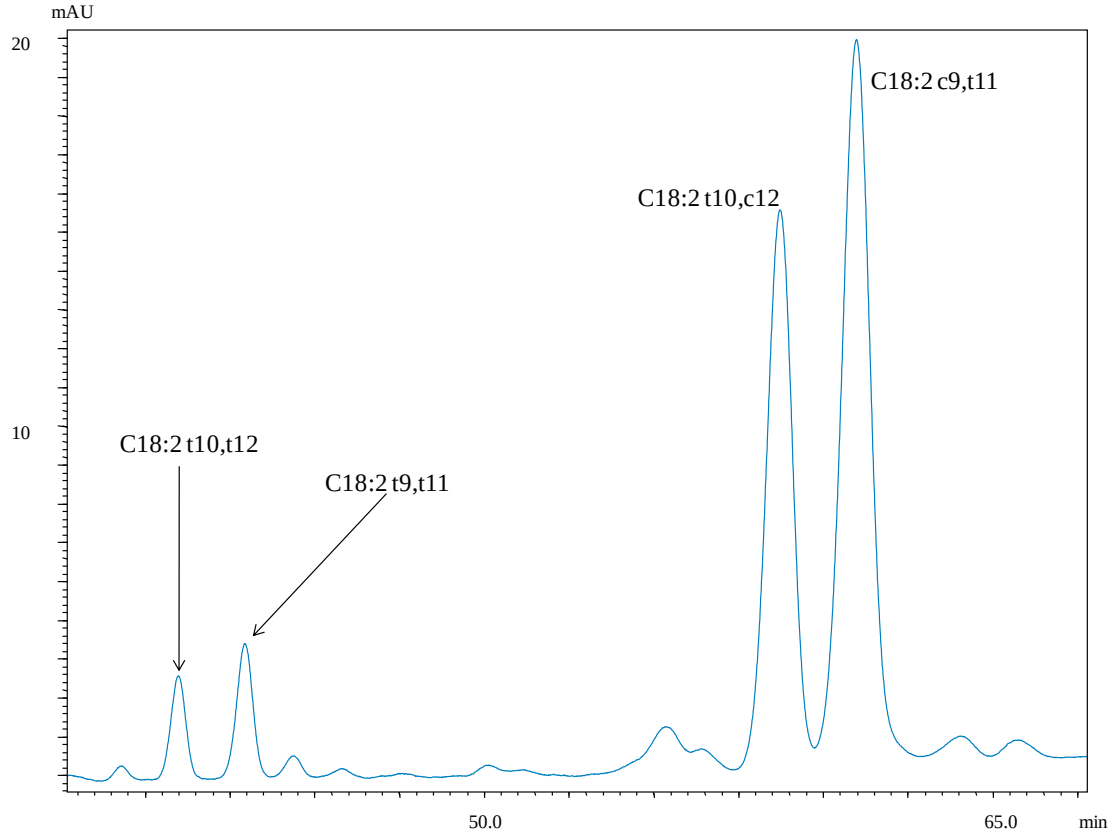

Fig. 3. Partial chromatogram of the CLA isomers profile assessed by Ag+-HPLC of the culture supernatant obtained of *L. Acidophilus lac1* in skim milk medium with free linoleic acid as substrate.

3.3. Evaluación del efecto de los tratamientos industriales convencionales (pasterización, UHT y esterilización) frente a los no convencionales (microondas, altas presiones y homogenización a alta presión) sobre el perfil lipídico y el contenido en fosfolípidos de leche de vaca, oveja y cabra.

Influence of heat treatments, high pressure and microwave processing of naturally PUFA enriched milk on CLA isomers distribution and *trans* fatty acids content

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Running title:

Keywords: CLA, milk, fatty acid composition, processing.

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Abbreviations: Ag^{+}-HPLC, silver-ion HPLC; RA, rumenic acid;

Abstract:

The possible effects of processing in the fatty acid composition of naturally CLA enriched milk have been studied in this research work. Two raw milk batches of 500 liters each, differenced in their atherogenic index (normal HMI, NH; enhanced HMI, HH) content as result of different cattle feeding, were processed to obtain pasteurization (72º 30”), HTST (85º, 30”), UHT-A (135º, 30”), UHT-B (150º 5´), sterilized (121º, 15´), high pressured (up to 400 MPa, 15´) and microwave heated milk (650 W, 1.30’). Fatty acid profile as well as CLA isomers, *trans* FA and the presence of oxidation radicals were analysed by GC, TLC, UV spectrophotometry, Ag^{+}-HPLC and DPPH assays respectively. Control HH milk showed a 290% increase ($p<0.05$) in the concentration of total CLA as well as increased concentrations of MUFA and *trans* vaccenic acid (TVA) were also observed as effect of feeding.

The processing applied to milk had no effects ($p>0.05$) on fatty acids composition of monounsaturated or saturated fatty acids. On the other hand HTST pasteurization conditions led to sigmatropic rearrangement of the CLA isomers, increasing the concentration of C18:2 *cis* 9, *trans* 11 instead sterilization processing caused the isomerization of linoleic acid towards C18:2 *trans* 9, *trans* 11. .

1. Introduction:

Due to the relatively high amount of saturated (SFA) and *trans* fatty acids in dairy fats and their impact on the risk to develop chronic (Nishida, Uauy, Kumanyika & Shetty, 2004) and other metabolic diseases (Haug, Hostmark & Harstad, 2007; Jacobson, Miller & Schaefer, 2007) food industries are being guided by recommendations to decrease saturated fatty acid and cholesterol contents as low as possible.

Nevertheless, in the last decades many investigations have reported that some milk fat compounds, as conjugated linoleic acid (CLA), are related to many biological activities and health benefits (Kanwar *et al.*, 2008; Parodi, 2004; Steijns, 2008).

Because of the greater sensitivity of milk fat to ruminant dietary manipulation, extensive work on enhancing CLA as well as other PUFA (as n-3 LCPUFA) has been done by supplementing forage/ensilage diets with highly PUFA oils or oilseeds, as well as with protected fats to resist biohydrogenation and enhance the concentration of PUFA in milk (Jenkins & McGuire, 2006).

Consequently, special attention should be taken when processing those highly enriched PUFA milks in order to avoid alterations of its nutritional value since PUFAs are prone to deterioration reactions such as oxidation, mainly when radical starters are present.

Although a high number of authors and reviews have reported stability of the total CLA content of enriched products as milk, yogurt and cheesses (Bisig, Eberhard, Collomb & Rehberger, 2007; Jones et al., 2005) when analyzing the isomers of CLA or *trans* fatty acids, some investigations found significative variations. Then in experiences carried out in milkfat and methyl linoleate at temperatures above 200ºC (Destaillats & Angers, 2002; Destaillats & Angers, 2005a), was reported that linoleic acid isomerize to C18:2

68 *trans* 9, *trans* 11 or C18:2 trans 10, trans 12 CLA and that into this fraction rumenic
69 acid through sigmatropic rearrangement reactions, isomerize to C18:2 *trans* 8 *cis* 10 and
70 *trans, trans* isomers as consequence of heating. Confirming this, in high CLA content
71 dairy powders (Rodríguez-Alcalá. & Fontecha, 2007) was reported that total *trans*,
72 *trans* CLA isomers concentration increased whereas total *cis cis* descent due to
73 atomization processing (150ºC). Campbell et al (2003) processed CLA enriched milk by
74 pasteurization reporting that rumenic acid but not C18:2 *trans* 10 *cis* 12 CLA
75 diminished within 24h after treatment and refrigerated storage. Herzallah et al (2005a;
76 2005b) conducted experiences with pasteurized, UHT, microwaving, yogurt and cheese.
77 In general fatty acid composition were not affected by processing but low pasteurization
78 (63ºC, 10 min) and microwaving treatments caused losses in total *trans* monoene fatty
79 acids.
80 Products alike to naturals are seeing by consumers as a preferable choice (Menrad,
81 2003; Urala & Lahteenmaki, 2007) and new emerging technologies as high pressure
82 technology are being studied to evaluate its potential as alternative or complementary
83 process to thermal pasteurization (Hayes, Fox & Kelly, 2005; Smiddy, Martin, Huppertz
84 & Kelly, 2007). Although have not been found modifications in the lipid fraction of
85 milk from different ruminant species (cow, goat and sheep) (Rodríguez-Alcalá, Harte &
86 Fontecha, 2009) either when processing by high pressures or high pressure
87 homogenization, it is known that those treatments can result in partial whey protein
88 denaturation, casein micelle dissociation, and disruption of the milk fat globule
89 membrane (MFGM) (Pereda, Ferragut, Quevedo, Guamis & Trujillo, 2007; Zamora,
90 Ferragut, Jaramillo, Guamis & Trujillo, 2007). There are also evidences that pressure <
91 100 MPa does not protect milk from enzymatic rancidity due to the lack of inactivation
92 of lipases (Datta, Hayes, Deeth & Kelly, 2005; Humbert, Driou, Guerin & Alais, 1980).

The aim of the present work is to study the possible effects of cow milk processing (thermal treatment or emerging technologies) and if these changes are influenced by PUFAs concentration.

2. Materials and methods

2.1 Chemicals

Hexane, Methanol and Chloroform were purchased from LabScan (Dublin, Ireland), Potassium hydroxide and Sodium sulphate-1 hydrate from Panreac (Barcelona, Spain), Conjugated Linoleic acid standards from Nu-Chek Prep (Elysian, Minnesota, USA), Rumenic acid, glyceryl tritridecanoate and 2,2-Diphenyl-1-picrylhydrazyl from Sigma (St. Louis, Missouri, USA), high CLA content oil (Tonalin®) from Cognis (Illertissen, Germany). All reagents used in these experiments were GC or HPLC grade. Reference milk fat butter BCR-164 (EU Commissions; Brussels, Belgium) was purchased from Fedelco Inc. (Madrid, Spain).

2.2 Samples

Two raw milk batches (500 liters each) differenced in order to their atherogenic index (Ulbricht & Southgate, 1991) as result of different cattle feeding (normal HMI, NH; high HMI, HH) were processed by duplicate obtaining Pasteurized (72º 30”), HTST (85º, 30”), UHT (UHT, 135º, 30” and UHT2, 150º 5´), Sterilized (121º, 15´), High pressure (400 MPa, 15´) and Microwave heated milk (650 W, 1.30’).

Pasteurized, HTST and UHT processes were performed in an aseptic sterilizer (Rossi&Catelli, Parma, Italy). Raw milk was also collected as control. Thirty liters of raw milk in every experience were preheated at 65º and then homogenized at 180 bars. Temperature was risen to pasteurization and then cooled to an output temperature of

20º. When processing UHT, a preheating stage (85º) was carried out after homogenization.

Sterilization of one liter of each batch (NH and HH) of raw milk was carried out in a lab autoclave (Selecta Autotester E DRY-PV, Barcelona, Spain). High pressure processing at 400 MPa, 15 min, 25ºC, took place in a lab-scale high pressure machine (ACB, GEC, Alsthom, Nantes, France) and using 200 mL of raw milk fitted in pressure resistant packages. Microwaving of 100 mL of raw milk was performed in a domestic apparatus (Texet Model 112).

In every case 1 liter of product was obtained and placed into a glass bottle with addition of Sodium Azida (0.06 $g \cdot mL^{-1}$) to avoid microbial growth.

2.3 Lipid extraction and fatty acid derivatization

Milk fat extraction was carried out according to standard methods (ISO, 2001). The fat residue extracted was collected into amber vials and stored frozen at –20ºC until analysis. FAMEs were prepared by base-catalyzed methanolysis of the triglycerides (2N KOH in methanol) according to ISO (2002). As internal Standard 1,2,3-Tritridecanoylglycerol (Sigma-Aldrich, Steinheim, Germany) was added to samples (200 μL, ,1,12 mg/mL).

2.4 Fractioning by $AgNO_3$-TLC of FAME

The FAMEs were fractionated according to the number and geometry of double bounds by TLC following the Precht and Molkentin procedure (Precht & Molkentin, 1996)

149 slightly modified. The TLC glass plates (20 ´ 20 cm) with silica gel (0.25 mm) (Merck,
150 Darmstadt, Germany) were incubated with 20% aqueous solution of AgNO3 (Panreac,
151 Barcelona, Spain) for 16 h, were partially air dried, and were activated at 120°C for 30
152 min. A 100-ml solution of FAME (80 mg/ml) was applied to the activated TLC glass
153 plate in a narrow band. The plate was developed twice in a saturated chamber in hexane
154 and diethyl ether (9:1, vol/vol) with 15-cm migration. At the end of chromatographic
155 runs, the plates were air dried and sprayed with a 0.20% ethanol solution of 2¢,7¢-
156 dichlorofluorescein, and the bands were visualized under UV light. The bands
157 corresponding to the saturated and *trans* monoenoic fatty acid methyl esters, which
158 were previously identified by a mixture of stearic fatty acid methyl ester (C18:0) and
159 elaidic fatty acid methyl ester (*trans*- 9-C18:1) (Sigma Chemical, St. Louis, MO)
160 running in AgNO3-TLC, were scraped into a flask. The FAMEs were extracted with 80
161 ml of diethyl ether in four extractions, and the solvent was evaporated in a rotary
162 evaporator and stream of nitrogen. The residue was dissolved in 200 ml of heptane and
163 used for GC analysis.
164
165 To calculate the total content of *trans*-C18:1 isomers, the ratio of C18:0 to total *trans*-
166 C18:1 was determined in the saturated plus *trans* monoenoic AgNO3-TLC fraction and
167 was related to the C18:0 content of total FAME. To calculate the total content of *trans*
168 C16:1 isomers, the ratio of total *trans*-C16:1 to *trans*-10-C18:1 plus *trans*-11-C18:1 was
169 determined in the *trans* monoenoic AgNO3-TLC fraction and related to the ratio of
170 C18:0 to *trans*-10-C18:1 plus *trans*-11-C18:1 in the saturated plus *trans* monoenoic
171 AgNO3-TLC fraction and to the C18:0 content of total FAME.
172
173

2.5 GC-FID analyses of Total and trans fatty acids.

A fast GC analysis on a VF-23, fused-silica capillary column (30 m x 0.25 mm i.d.x 0.25 μm film thickness, Varian, Middelburg, Netherlands), was developed. The FAME were analysed on a Clarus® 500 chromatograph (Perkin Elmer, Beaconsfield, UK). The column was held at 120ºC for 1 min after injection, temperature-programmed at 10ºC/min to 140ºC, then temperature-programmed at 15ºC/min to 180ºC and last ramp at 5ºC to 240ºC held there for 3 min. Helium was the carrier gas with a column inlet pressure set at 10 psig and a split ratio of 1:20. The injection volume was 0.5 μl. Total run time was of 15 min.

On the other hand, for a more exhaustive identification of the fatty acids and to achieve a more complete lipid profile, a second chromatographic system besides the former described above, was used on a Perkin-Elmer chromatograph (model Autosystem, Beaconsfield, UK) with a FID detector. Fatty acids methyl esters were separated using CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, Netherlands). The column was held at 100º C for 1 min after injection, temperature-programmed at 7º C/min to 170º C, held there for 55 min, then temperature-programmed at 10º C/min to 230º C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 214 KPa (30 Psig) and a split ratio of 1: 20. The injection volume was 0.5 μl.

Response factors were calculated as obtained from a certified reference butter fat (CBR-164, EU Commissions; Brussels, Belgium, purchased to Fedelco Inc., Madrid, Spain) and used for samples peak area correction.

199

200 *2.6 Silver-ion HPLC (Ag^+-HPLC)*

201

202 Ag^+-HPLC separation of CLA methyl esters was arranged using a HPLC (Shimadzu
203 Vp Series, Duisburg, F.R. Germany) equipped with UV detector operated at 233 nm and
204 a ChromSpher 5 Lipid analytical column (4.6 mm i.d. X 250 mm stainless steel; 5 μm
205 particle size; Varian, Middelburg, Netherlands). The mobile phase was 0.1% acetonitrile
206 in hexane, operated isocratically at a flow rate of 1.0 mL/min. The injection volume was
207 10μl. As standard a mixture of pure CLA FAME isomers from Nu-Chek Prep. Inc.
208 (Elysian, MN, USA) was used.

209

210 *2.7 Quantification of total CLA content by UV spectroscopy*

211

212 Total CLA determination was carried out according to Bernuy et al.(2008) as follows:
213 samples were weighted to obtain a concentration of 0.19 mg/ mL in hexane; 2-mL were
214 placed in quartz cuvettes, and absorbance the was recorded at 234 nm against pure
215 hexane as blank, in a Perkin-Elmer spectrophotometer (Lambda650 model,
216 Beaconsfield, UK) with a scan program (190-350 nm).
217 The concentration of CLA was determined by means of a calibration curve as follows:
218 A solution of Rumenic acid in hexane containing 0.746 mg/mL was diluted at different
219 concentration (R^2=0,9636), measured in triplicate and used to calculate the total CLA
220 concentration of a high CLA commercial oil (79,4%). A new calibration curve, using
221 this oil, was accomplished with concentrations ranged from 0 to 0.02 mg CLA/mL
222 (R^2=0,9989).

223

224 *2.8 Antiradical scavenging DPPH assays*

225

226 To evaluate the effects of milk processing on the antioxidants and the possible
227 formation of radicals an antiradical scavenging assay using DPPH was accomplished
228 according to Brand-Williams et al (1995) with slightly modifications: 50 mg of sample
229 was dissolved in 200 μL of trichloromethane. 400 μL of DPPH solution (0.09 mg/mL)
230 was added and the mixture incubated overnight. Absorbance at 515 nm was measure in
231 a Perkin-Elmer spectrophotometer (Lambda650 model, Beaconsfield, UK) and the
232 results expressed as percentage of inhibition of DPPH against a blank preparation (200
233 μL of trichloromethane plus 400 μL of DPPH). The acceptability and linearity of the
234 concentrations and solvent used was tested by means of a calibration curved using the
235 DPPH solution in a range of concentrations from 0 to 0.06 mg/mL (R^2=0.9986). All
236 measures were performed in triplicate.

237 *2.9 Statistical analysis*

238

239 Full factorial data analysis (one way Anova and GLM) were conducted with the aid of
240 the SPSS package (SPSS 15.0 for Windows, SPSS Inc.).

241

3. Results and discussion

3.1 Effect of feeding in the fatty composition of cow milk

The fatty acid composition of the milks used in this study showed different atherogenic index (HMI; MUFAS+PUFAS/C12+4*C14+C16) (Bobe, Hammond, Freeman, Lindberg & Beitz, 2003; Ulbricht et al., 1991) due to feeding of cows. Samples labeled as NH (normal HMI) had a HMI of 0.43 instead of 0.61 in HH milk (enhanced HMI).

Basic parameters were determined by means of Milkoscan: fat content (2,74±0,07 in samples HH and 3,53±0,21 in NH), protein (3,13±0,05, 3,15±0,01), lactose (5,09±0,81, 4,71±0,07) and dried extract (11,33±0,23, 14,62±0,29).

The differences according to the fat content can be explained taking into account that the incorporation of oils and fats in the diets of the cattle changes the relation fiber starch, directing the metabolic routes of the microorganisms of the rumen to acid fermentations, so that the production of acetate diminishes and therefore influences the synthesis of milkfat (Schroeder, Gagliostro, Bargo, Delahoy & Muller, 2004).

The analyses realized by GC (Tables 1 and 2) showed that the total content of saturated fatty acids is significantly minor in the group of samples HH (58.5 %) that in NH (66.38 %). The major fatty acids of this fraction (palmitic, miristic and lauric acids decreased its contents ($p<0.05$) when comparing with the composition of the cows feds the control diet. On the contrary, stearic acid concentration significantly increased as effect of feeding (9.13 %, 10.21 %).

These results agree with other previously reported, where the inclusion of certain fatty acids sources (oils rich in linoleic) in the rations feed to the cows led to lower contents

of medium chain fatty acids and higher concentrations of palmitic, linoleic and Oleic in milk (Lawless, Murphy, Harrington, Devery & Stanton, 1998; Rego et al., 2005).

This may be due to the change in pH of the rumen, which inhibits the fatty acids lipolysis, primarily of Stearic acid and Myristic (Demeyer & Doreau, 1999). As well as de novo synthesis of these fatty acids in mammary gland from acetate would be affected by the acid fermentation (Jensen, 2002; McNamee, Fearon & Pearce, 2002).

MUFAs (33.11% 27.58% in HH vs. NH) increased ($p<0.05$) in the milk as effect of the diet. Within the compounds of this group, there has been an increase ($p<0.05$) of Oleic acid (21.91% vs. HH. NH 20.64%). In the total content of C18:1 *cis* fatty acids rose its content significantly from 1.37% in the samples NH to 2.38% in HH by the higher concentrations of C18: *cis* 11, C18: 1 *cis*-12 and C18: 1 *cis* 13. The higher contents of C18: 1 *cis*-9 is related to the activity of Δ^9-desaturase, acting on stearic, although it has proposed its action on other saturated fatty acids (Jenkins et al., 2006).

As with the compounds of *cis* octadecene, the content of trans fatty acids show a significant increase in the samples HH (5.63% vs. 3.11%). In this group of compounds the highest increases were registered for the C18: 1 trans 10 (20.71 mg • g-1 vs. 4.35 mg • g-1) and vaccenic (14.6 mg • g-1 vs. 8.73 mg • g-1) as can be seen in tables 3-6 ($p<0.05$).

This behavior is widely described in the literature that investigated the effects of ruminant feed in the lipid composition of milk. Is accepted that feeding, by altering the metabolic pathways of the rumen, increases both vaccenic as C18:1 *trans* 10 (Elgersma, Tamminga & Ellen, 2006; Li, Wang, Li & Lin, 2006).

The biohydrogenation of fatty acid by the rumen bacteria is produced from linoleic and linolenic of the diet and resulting in the production of rumenic, vaccenic and stearic

292 acids (Tanaka, 2005). However theses processes do not completely explain the presence
293 of fatty acids in milk fat although similar pathways for other compounds have been
294 suggested (Parodi, 2004).
295
296 Coinciding with the effects on total monounsaturated fatty acids, polyunsaturated
297 increased ($p<0.05$), from 4.42% in raw milk of NH to 6.42% in HH. Linoleic (6.42% vs.
298 4.42%) was significantly and positively affected by feeding as total conjugated linoleic
299 acid doubled its concentration in HH samples (Tables 1, 2 and 9). The results in tables 1
300 2, 7 and 8 exhibit that rumenic acid (C18: 2 *cis*-9 *trans* 11) was increased significantly
301 from 0.39% (3.64 mg • g-1) in NH group to 0.84% (7.77 mg • g-1) in HH, besides being
302 the main compound in the CLA fraction as well as C18: 2 *cis* 7 *trans* 9 (0.44 mg • g-1 in
303 NH vs 1.03 mg g-1 in HH).
304
305 These results can be explained as effect of diet on the composition of milk fat due to
306 linoleic acid obtained through diet is biohydrogenated in the rumen (Dhiman, Seung-
307 Hee & Ure, 2005). Likewise vaccenic acid produced in the rumen and reaching the
308 mammary gland will be *trans*formed by Δ^9-desaturase into rumenic, reason why the HH
309 samples show higher contents of total CLA and RA.
310
311 The data exposed in this research work is supported by other previous experiences
312 where it was reported that supplementation with different lipid sources is a useful way
313 towards obtaining higher conjugated linoleic acid content in milk (Loor, Ferlay, Ollier,
314 Doreau & Chilliard, 2005; Nudda, Battacone, Usai, Fancellu & Pulina, 2006). Certain
315 investigations suggested the possibility that increased certain isomers of CLA, in

316 particular C18: 2 *trans*-10 *cis* 12, are responsible for the decrease in fat content of milk
317 (Lock et al., 2007).
318 This is a point on which there is broad debate, since many experiences studying the
319 effects of supplementation in the feeding of cows, despite improving the content
320 MUFAs and PUFAs, induced an increase of some undesirable compounds for their
321 effect on human health (Soustre, Laurent, Schrezenmeir, Pfeuffer, Miller & Parodl,
322 2002; Woodside & Kromhout, 2005), as the C18: 1 *trans* 10, which is also believed to
323 cause lower milkfat contents (Gaynor, Waldo, Capuco, Erdman, Douglass & Teter,
324 1995; Loor, Ferlay, Ollier & Chilliard, 2005).
325
326
327 *3.2 Effect of processing in the fatty acid composition*
328
329 All samples were processed to obtain pasteurized, UHT, sterilized, microwave and high
330 pressure milks. Due to the feeding of the cows, one of the milk had a higher content in
331 PUFAs. The experiment was designed to test if milkfat is affected by processing and
332 changes associated to the fatty acid composition.
333
334 The existing literature related to this point is scarce. Herzallah et al. (2005a) processed
335 milk, obtaining pasteurized, UHT, boiled and microwave boiled milks and these
336 treatments had not effect on the total contents of saturated, monounsaturated and
337 polyunsaturated fatty acids concluding that due to the protective action of the
338 continuous aqueous phase, acts as an oxygen barrier that hinders the oxidation process,
339 triglycerides are stable during heating processing.

In previous research (Jones et al., 2005) cows feed with a normal and an experimental diet, showing the latter a more unsaturated milkfat. The effect of heating treatment was investigated and both milks were processed to obtain UHT milk. The detailed composition in fatty acids was reported, as well as no changes in agreement with those results of Herzallah et al (2005a). In a similar assay Lynch et al (2005) did not also found changes in pasteurized milk from milks with enhanced-by-feeding levels of vaccenic acid and PUFAs.

The results obtained in the current research work did not find that the saturated and monounsaturated fatty acids were affected when milks were treated to obtain pasteurized, UHT, sterilized, microwave and high pressures milks as well as not was detected a distinctive behavior attributable to the fact that a group of samples had a more polyunsaturated fat. Nevertheless both groups of samples showed a trend toward slightly lower concentrations of linoleic acid in the processed samples than in control milks.

In this investigation were performed analysis of TFAs by TLC and also were obtained results by applying gas chromatography. As in the general composition of fatty acids, the distribution of these compounds was not altered by processing. Previous works according to the effect of processing of milk in the *trans* fatty acids (Herzallah et al., 2005b) conclude that pasteurization (63ºC, 30 min) and microwave (95.8ºC, 10 min) can increase the total content of TFA in milk. This mentioned study do not report a general composition of the fatty acids where it could be seem the specific moieties affected by treatments as well as the source of increase. Authors explain their results as effect of oxidative reactions.

Our results show that *trans* fatty acids are stable compounds even when the contents of monounsaturated and PUFAs has been enhanced by feeding. Other minor compounds as C18:2 isomers (not conjugated) did not also were altered when both milks were processed.

Precht et al.(1999) assayed heating of buttermilk at high temperatures (20-350ºC) resulting in isomerization of oleic in elaidic acid and losses in linoleic acid, rumenic acid and linolenic explained through oxidation reactions. Others authors reported that heating of vegetable oils above 200ºC isomerizes linoleic acid into CLA isomers (Juaneda, de la Perriere, Sebedio & Gregoire, 2003).

The reaction mechanism in the transformation of linoleic acid to CLA involves the presence of radicals when the content of these fatty acids is of 20-30% and direct isomerization in lower concentrations (Destaillats et al., 2005a). In milk fat has been also reported that heating can alter the distribution of the CLA isomers due to the sigmatropic rearrangement of Rumenic and C18:2 *trans* 10, *cis* 12 to *trans*, *trans* CLA compounds (Destaillats, Japiot, Chouinard, Arul & Angers, 2005b).

Some experiences conducted in fortified milks and dairy products with high CLA contents (Campbell et al., 2003; Rodriguez-Alcala & Fontecha, 2007) reported different conclusions: Campbell et al (2003) found that pasteurization affect the content of rumenic, diminishing its concentration and Rodriguez and Fontecha (2007) reported that in CLA enriched dairy products thermal treatments resulted in higher contents of *trans*, *trans* and *cis*, *trans*-*trans*, *cis* isomers while total contents kept unchanged pointing to sigmatropic rearrangement reactions of the CLA fraction. In milk, the effect of thermal processing on the total CLA content was reported by Herzallah et al (2005b) where

389 microwave and UHT processing resulted in lower contents of conjugated linoleic acid
390 isomer.
391
392 In the present work when NH and HH milks were processed to obtain sterilized milk,
393 the concentration of C18:2 *trans* 9, *trans* 11 increased ($p<0.05$) in agreement with the
394 work of Angers and Destaillats (2005a) where the formation of this fatty acid was
395 thermally induced as a product of the isomerization of linoleic acid.
396 However the total CLA content obtained applying UV spectrophotometry (Table 9.) to
397 the samples shows that the total concentrations of this fatty acids do not resulted
398 increased ($p>0.05$) when sterilizing. In the other hand linoleic acid showed a trend to
399 increase its concentration.
400
401 The content of rumenic acid was higher ($p<0.05$) when HH milk was processed by
402 microwave HTST (Table 8). This agree with the total content of CLA (Table 9) that was
403 increased when HH milk were processed by HTST treatments ($p<0.05$).
404
405 Low temperature treatments create oxidative environments due to oxygen has lower
406 solubility at this temperatures, H^+-proton donors proteins (very sensible to oxidation)
407 are denaturated in less extension than in other treatments and Maillard compounds
408 (some reported as antioxidant) are hardly formed (Giroux, St-Amant, Fustier, Chapuzet
409 & Britten, 2008). Our results according to the above mentioned research works point
410 out that oxidative reactions are taking account in these samples when processing by
411 pasteurization and microwave, favored by the presence of proton donors. Other
412 publications elsewhere (Ferreri, Costantino, Perrotta, Landi, Mulazzani &
413 Chatgilialoglu, 2001; Geißler, Brede & Reinhardt, 2003) have described that

polyunsaturated fatty acids can be isomerized into *cis*/*trans* compounds; this catalyzed by thiyl radicals formed from thiols groups. According to all this, the higher presence of oxygen in the pasteurization and microwave processing of milk leads to the formation of radicals from proteins which could react with linoleic acid increasing the concentrations of rumenic acid.

The dpph assays (data not showed) reveal in both groups of samples a tendency ($p>0.05$) toward a higher inhibition of the 2,2-Diphenyl-1-picrylhydrazyl; due to this compound can react with radicals formed during oxidation. The % of inhibition was found as not affected by the unsaturated fatty acid contents.

To sum up, according to processing, there are two effects focused in the CLA isomers, independent of the fat composition: the first take place in pasteurization (HTST) and microwaving `processing and resulting in higher contents of Rumenic acid by sigmatropic rearrangement of the compounds of the conjugated Linoleic acid fraction.

In the second, is involved oxidation and high temperature conditions (sterilization processing) which catalyzed the isomerization of Linoleic acid into C18:2 *trans* 9, *trans* 11.

438

439

440 **Tables:**

441 **Table 1.**

442 Fatty acid content (g / 100 g FA) in milks with normal HMI (NH).

Fatty acid	Raw	Past.	HTST	UHT	UHT2	Estr.	HP	Micro.	P
C4	3.63	3.14	3.08	3.02	2.82	3.20	3.14	3.31	0.31
C6	2.32	1.98	2.19	2.11	2.11	2.11	2.12	2.40	0.67
C8	1.27	1.39	1.23	1.24	1.30	1.30	1.20	1.23	0.05
C10	2.81	2.87	2.75	2.77	2.97	2.85	2.65	2.73	0.87
C10:1	0.31	0.33	0.30	0.30	0.33	0.31	0.28	0.30	0.95
C12	3.06	3.26	3.10	3.10	3.26	3.11	3.05	3.08	0.70
C14	9.92	10.26	10.24	10.26	10.08	10.02	10.06	10.16	0.39
C15i	0.20	0.21	0.21	0.21	0.21	0.20	0.20	0.21	0.56
C15ai	0.84	0.46	0.46	0.47	0.46	0.39	0.45	0.46	0.30
C14:1	0.45	0.88	0.87	0.87	0.88	0.85	0.85	0.86	0.68
C15	1.07	1.11	1.11	1.09	1.11	1.08	1.10	1.10	0.27
C16i	0.20	0.21	0.21	0.21	0.19	0.20	0.21	0.21	0.63
C16	32.09	31.71	31.96	32.18	32.78	31.83	32.09	31.93	0.94
C17i	0.20	0.27	0.27	0.28	0.28	0.26	0.25	0.24	0.70
C16:1	1.85	1.86	1.86	1.88	1.92	1.81	1.82	1.86	0.54
C17	0.54	0.52	0.53	0.53	0.54	0.53	0.53	0.53	0.89
C17:1	0.24	0.25	0.25	0.25	0.25	0.25	0.26	0.24	1.00
C18	9.13	8.96	9.11	9.26	9.50	9.11	9.18	9.14	0.82
C18:1 t	2.19	2.10	2.06	2.11	1.83	2.11	2.02	2.03	0.95
C18:1 c9	20.64	20.61	20.40	20.17	19.39	20.66	20.78	20.42	0.37
C18:1 c	1.37	1.41	1.48	1.44	1.25	1.44	1.37	1.33	0.76
C18:2 other	0.63	0.57	0.59	0.59	0.54	0.62	0.61	0.57	0.32
C18:2 c9,c12	2.64	2.45	2.48	2.43	2.34	2.53	2.54	2.46	0.26
C20	0.13	0.11	0.12	0.11	0.11	0.13	0.12	0.12	0.85
C20:1	0.14	0.15	0.16	0.16	0.15	0.17	0.16	0.10	0.70
C18:3	0.31	0.33	0.33	0.33	0.30	0.32	0.33	0.33	0.17
C18:2 CLA	0.39	0.42	0.43	0.43	0.39	0.41	0.41	0.43	0.09
CLA otros	0.14	0.13	0.14	0.13	0.13	0.13	0.14	0.14	1.00
C22	0.04	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.53
C20:3	0.12	0.14	0.14	0.13	0.13	0.12	0.13	0.14	0.37
C20:4 AA	0.19	0.20	0.21	0.20	0.19	0.19	0.20	0.20	0.76
Total N.i	1.62	1.65	1.68	1.70	1.91	1.67	1.71	1.72	1.00
Σ SCFA	6.52	6.51	6.50	6.36	6.23	6.60	6.46	6.94	0.82
Σ MCFA	18.87	19.59	19.25	19.27	19.78	19.02	18.86	19.10	0.67
Σ LCFA	72.99	72.25	72.57	72.67	72.08	72.70	72.97	72.24	0.79
Σ SFA	66.38	66.94	67.02	67.26	68.49	66.85	66.80	67.28	0.33
Σ MUFA	27.58	27.17	26.99	26.79	25.58	27.15	27.15	26.73	0.56
Σ PUFA	4.42	4.24	4.31	4.26	4.02	4.32	4.35	4.27	0.13
Total 18:1	22.01	22.02	21.88	21.61	20.64	22.10	22.15	21.75	0.44
Total *trans*	3.11	2.10	2.06	2.11	1.83	2.79	2.02	2.03	0.08
Total 18:2	3.28	3.02	3.07	3.02	2.88	3.16	3.14	3.03	0.07
Total CLA	0.53	0.56	0.57	0.57	0.52	0.54	0.55	0.57	0.47

443 Abbreviations: *trans* (t), cis (c), arachidonic acid (AA).

444

445 **Table 2.**

446 Fatty acid content (g /100 g FA) in milks with improved HMI (HH).

Fatty acid	Raw	Past.	HTST	UHT	UHT2	Estr.	HP	Micro.	P
C4	3.29	2.94	2.74	2.93	3.05	2.98	2.86	2.80	0.67
C6	2.12	1.84	1.79	1.84	1.88	1.91	1.81	1.83	0.27
C8	1.11	0.97	1.08	0.99	1.05	1.11	1.04	1.09	0.05
C10	2.37	2.17	2.39	2.20	2.31	2.41	2.27	2.37	0.03
C10:1	0.24	0.21	0.24	0.22	0.23	0.24	0.22	0.21	0.83
C12	2.64	2.57	2.71	2.55	2.64	2.66	2.61	2.67	0.04
C14	9.22	9.33	9.63	9.21	9.40	9.38	9.34	9.54	0.03
C15i	0.13	0.16	0.17	0.16	0.16	0.17	0.15	0.17	0.43
C15ai	0.83	0.84	0.86	0.83	0.84	0.89	0.86	0.86	0.30
C14:1	0.42	0.43	0.44	0.38	0.43	0.39	0.43	0.44	0.65
C15	1.02	1.03	1.06	1.02	1.04	1.04	1.03	1.06	0.46
C16i	0.19	0.17	0.21	0.19	0.20	0.18	0.20	0.20	0.57
C16	24.64†	25.25	25.73	25.01	25.27	25.31	25.13	25.68	0.20
C17i	0.31	0.29	0.32	0.32	0.32	0.23	0.32	0.32	0.56
C16:1	1.65	1.69	1.72	1.67	1.68	1.67	1.68	1.72	0.05
C17	0.55	0.57	0.58	0.56	0.57	0.57	0.57	0.58	0.08
C17:1	0.27	0.28	0.26	0.26	0.26	0.25	0.26	0.26	0.80
C18	10.21†	10.47	10.61	10.43	10.51	10.52	10.45	10.65	0.57
C18:1 t	5.63†	5.68	5.50	5.75	5.54	5.49	5.65	5.39	0.97
C18:1 c9	21.91†	22.01	21.43	22.12	21.64	21.49	22.01	21.38	0.24
C18:1 c	2.38†	2.31	2.12	2.30	2.26	2.13	2.42	2.19	0.17
C18:2 otros	1.02†	0.93	0.95	0.99	0.97	0.93	1.02	0.95	0.59
C18:2 c9,c12	3.81†	3.74	3.56	3.62	3.61	3.49	3.68	3.56	0.48
C20	0.13	0.12	0.11	0.12	0.11	0.11	0.12	0.11	0.92
C20:1	0.20	0.09	0.09	0.19	0.17	0.18	0.09	0.18	0.33
C18:3	0.34	0.35	0.36	0.34	0.35	0.35	0.34	0.36	0.11
C18:2 CLA	0.84†	0.85	0.88	0.84	0.85	0.85	0.85	0.88	0.09
CLA otros	0.18	0.18	0.19	0.18	0.18	0.18	0.18	0.19	0.88
C22	0.05	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.66
C20:3	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.13	0.23
C20:4 AA	0.18	0.18	0.19	0.18	0.18	0.18	0.18	0.19	0.84
Total N.i	1.97	2.14	1.91	2.37	2.11	2.53	2.04	2.00	0.59
Σ SCFA	6.52	5.75	5.61	5.76	5.97	6.00	5.71	5.72	0.25
Σ MCFA	17.07†	16.93	17.71	16.77	17.26	17.35	17.10	17.52	0.05
Σ LCFA	74.44	75.18	74.78	75.09	74.66	74.12	75.15	74.77	0.45
Σ SFA	58.50†	58.38	59.61	57.99	59.00	59.02	58.38	59.56	0.12
Σ MUFA	33.11†	33.13	32.21	33.35	32.61	32.35	33.21	32.18	0.58
Σ PUFA	6.42†	6.36	6.26	6.29	6.27	6.11	6.37	6.26	0.74
ΣTotal 18:1	29.92	30.00	29.04	30.18	29.43	29.11	30.08	28.96	0.51
Total *trans*	5.63†	5.68	5.50	5.75	5.54	5.49	5.65	5.39	0.97
Total 18:2	4.83†	4.67	4.51	4.61	4.58	4.42	4.69	4.51	0.43
Total CLA	1.01†	1.03	1.06	1.02	1.03	1.03	1.03	1.07	0.26

447 † significant differences by feeding ($p<0.05$). Abbreviations: *trans* (t), *cis* (c), arachidonic acid (AA).

448

449 **Table 3.**

450 C18:1 and C18:2 contents (g/ 100 g FA) in milks with normal HMI (NH).

Fatty acid	Raw	Past.	HTST.	UHT.	UHT2.	Est.	HP.	Micro.	P
C16:1 t	0.16	0.17	0.16	0.16	0.17	0.16	0.16	0.16	0.51
C18:1 6-8t	0.31	0.27	0.29	0.27	0.25	0.28	0.29	0.28	0.72
C18:1 9t	0.25	0.23	0.29	0.24	0.22	0.30	0.26	0.24	0.86
C18:1 10t	0.46	0.43	0.42	0.43	0.39	0.44	0.44	0.49	0.70
C18:1 11t VA	0.80	0.76	0.79	0.77	0.71	0.79	0.83	0.69	0.64
C18:1 12-13t + 7c	0.40	0.37	0.41	0.37	0.34	0.43	0.39	0.28	0.34
C18:1 11c	0.68	0.64	0.65	0.65	0.60	0.68	0.70	0.66	0.38
C18:1 12c	0.35	0.31	0.32	0.32	0.29	0.33	0.33	0.32	0.55
C18:1 13c	0.08	0.07	0.06	0.07	0.06	0.08	0.08	0.07	0.07
C18:1 14c+16t	0.28	0.27	0.28	0.26	0.23	0.30	0.30	0.28	0.17
C18:1 15c	0.07	0.07	0.08	0.08	0.06	0.09	0.08	0.08	0.34
C18:2 t,t NMID	0.24	0.20	0.21	0.22	0.19	0.23	0.22	0.22	0.14
C18:2 t9,t12	0.11	0.09	0.10	0.10	0.08	0.10	0.10	0.09	0.07
C18:2 c9,t13	0.11	0.08	0.09	0.08	0.08	0.09	0.09	0.09	0.08
C18:2 c9,t12	0.08	0.02	0.08	0.08	0.07	0.09	0.09	0.07	0.07

451 Abbreviations: *trans* (t), *cis* (c), *trans* vaccenic acid (VA).

452

453 **Tabla 4. C18:1 and C18:2 contents (g/ 100 g FA) in milks with improved HMI (HH).**

Fatty acid	Raw	Past.	HTST	UHT	UHT2	Est.	HP	Micro.	P
C16:1 t	0.16†	0.16	0.17	0.16	0.16	0.16	0.17	0.17	0.38
C18:1 6-8t	0.60†	0.61	0.55	0.61	0.58	0.58	0.61	0.55	0.45
C18:1 9t	0.48†	0.50	0.42	0.50	0.48	0.48	0.49	0.43	0.25
C18:1 10t	2.32†	2.33	2.16	2.36	2.24	2.26	2.34	2.19	0.48
C18:1 11t VA	1.82†	1.90	1.68	1.90	1.83	1.77	1.86	1.63	0.10
C18:1 12-13t + 7c	0.85†	0.86	0.79	0.88	0.85	0.82	0.87	0.79	0.27
C18:1 11c	0.79†	0.83	0.78	0.83	0.81	0.76	0.86	0.80	0.39
C18:1 12c	0.89†	0.95	0.84	0.90	0.88	0.84	0.76	0.79	0.27
C18:1 13c	0.11†	0.09	0.06	0.11	0.09	0.10	0.10	0.06	0.32
C18:1 14c+16t	0.46†	0.44	0.32	0.45	0.45	0.43	0.46	0.41	0.16
C18:1 15c	0.14	0.14	0.11	0.13	0.15	0.14	0.15	0.14	0.12
C18:2 t,t NMID	0.46†	0.43	0.40	0.41	0.41	0.38	0.42	0.40	0.32
C18:2 t9,t12	0.17†	0.15	0.14	0.14	0.14	0.13	0.15	0.14	0.39
C18:2 c9,t13	0.17†	0.15	0.14	0.15	0.13	0.13	0.15	0.14	0.35
C18:2 c9,t12	0.14†	0.13	0.13	0.13	0.14	0.12	0.13	0.12	0.50

454 † significant differences by feeding. Abbreviations: *trans* (t), *cis* (c), *trans* vaccenic acid (VA).

455

456

457 **Tabla 5.**

458 *Trans* fatty acids (mg TFA/ g Fat) in milks with normal HMI (NH).

Fatty acid	Raw	Past.	HTST	UHT	UHT2	Est.	HP	Micro.	P
16:1 t	1,36	1,42	1,34	1,42	1,47	1,36	1,35	1,40	0,33
C18:1 4t	0,32	0,30	0,30	0,28	0,32	0,31	0,30	0,29	0,27
C18:1 5t	0,34	0,36	0,32	0,38	0,37	0,37	0,31	0,30	0,24
C18:1 6-8t	2,34	2,17	2,52	2,77	2,64	2,76	2,84	2,78	0,31
C18:1 9t	2,81	3,07	2,58	2,74	2,41	3,25	3,60	2,45	0,10
C18:1 10t	4,35	4,64	5,17	4,25	4,13	4,93	5,13	5,17	0,08
C18:1 11t VA	8,73	8,24	8,51	7,82	6,88	8,27	8,03	7,92	0,05
C18:1 12t	3,80	3,34	3,96	2,93	3,01	2,58	3,62	3,54	0,08
C18:1 13-14t	7,69	6,50	5,66	7,41	4,47	5,22	5,41	6,12	0,14
C18:1 15t	2,25	2,39	2,32	2,58	2,39	2,13	2,31	2,38	0,47
C18:1 16t	2,38	2,70	2,34	2,36	2,59	2,28	2,49	2,55	0,16

459 Abbreviations: *trans* (t), *cis* (c), *trans* vaccenic acid (VA).

460

461 **Tabla 6.**

462 *Trans* fatty acids (mg TFA/ g Fat) in milks with improved HMI (HH).

Fatty acid	Raw	Past.	HTST	UHT	UHT2	Est.	HP	Micro.	P
16:1 t	1.45	1.44	1.51	1.49	1.52	1.43	1.53	1.55	0.08
C18:1 4t	0.47	0.52	0.45	0.42	0.47	0.44	0.48	0.43	0.40
C18:1 5t	0.48	0.54	0.45	0.47	0.50	0.46	0.49	0.47	0.29
C18:1 6-8t	5.46†	5.02	4.74	4.95	5.06	4.78	4.73	4.39	0.11
C18:1 9t	5.60†	5.51	4.90	5.58	5.02	4.83	4.82	4.64	0.09
C18:1 10t	20.71†	20.88	20.08	21.70	21.33	19.96	19.85	19.73	0.47
C18:1 11t	14.60†	15.05	14.28	14.84	14.50	14.11	14.63	14.13	0.10
C18:1 12t	5.49†	6.48	5.83	5.63	6.23	6.47	6.22	6.07	0.21
C18:1 13-14t	8.16†	9.26	9.87	10.85	11.00	9.04	9.90	10.50	0.10
C18:1 15t	3.76†	4.76	3.35	3.78	4.58	4.66	4.86	4.22	0.10
C18:1 16t	4.22†	5.32	3.56	3.60	4.99	4.53	4.77	4.54	0.16

463 † significant differences by feeding. Abbreviations: *trans* (t), *cis* (c), *trans* vaccenic acid (VA).

464

465 **Tabla 7.**

466 CLA isomers (mg/g FA) in milks with normal HMI (NH).

Isomer	Raw	Past.	HTST	UHT	UHT2	Est.	HP	Micro.	P
trans, trans	0.34	0.40	0.44	0.41	0.38	0.43	0.39	0.40	0.27
12,14	0.04	0.04	0.05	0.04	0.05	0.04	0.04	0.04	0.63
11,13	0.06	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.85
10,12	0.10	0.11	0.11	0.11	0.09	0.11	0.10	0.10	0.23
9,11	0.06[a]	0.08[ab]	0.10[ab]	0.10[ab]	0.09 [ab]	0.11 [b]	0.08 [ab]	0.08 [ab]	0.00
8,10	0.06	0.06	0.08	0.07	0.06	0.08	0.07	0.07	0.05
7,9	0.03	0.04	0.04	0.04	0.03	0.04	0.04	0.04	0.43
cis, trans-trans,cis	4.13	4.84	4.97	4.91	4.23	4.54	4.72	4.93	0.26
12,14	0.02	0.02	0.02	0.04	0.03	0.03	0.03	0.03	0.14
11,13	0.04	0.03	0.03	0.04	0.03	0.04	0.05	0.04	0.49
9,11	3.64	4.27	4.38	4.31	3.71	3.98	4.14	4.33	0.23
7,9	0.44	0.51	0.53	0.52	0.45	0.50	0.51	0.53	0.40

Bold numbers used for significant changes by processing.

Tabla 8.

CLA isomers (mg/g FA) in milks with improved HMI (HH).

Isomer	Raw	Past.	HTST	UHT	UHT2	Est.	HP	Micro.	P
trans, trans	0.34	0.39	0.43	0.40	0.44	0.49	0.43	0.46	0.18
12,14	0.05	0.06	0.07	0.06	0.07	0.06	0.07	0.07	0.36
11,13	0.07	0.07	0.08	0.07	0.08	0.08	0.09	0.09	0.22
10,12	0.10	0.11	0.11	0.09	0.10	0.11	0.11	0.14	0.07
9,11	0.06[a]	0.07 [a]	0.09 [ab]	0.10 [ab]	0.11 [ab]	0.13 [b]	0.08 [a]	0.08 [ab]	0.00
8,10	0.03	0.04	0.05	0.04	0.04	0.06	0.05	0.04	0.34
7,9	0.03	0.03	0.04	0.04	0.05	0.06	0.04	0.04	0.05
cis, trans-trans,cis	8.88[†, ac]	9.13 [a]	10.06 [bc]	9.60 [a]	9.50 [abc]	9.44 [abc]	9.19 [abc]	10.15 [b]	0.00
12,14	0.04	0.04	0.04	0.03	0.04	0.04	0.04	0.05	0.54
11,13	0.04	0.03	0.04	0.04	0.05	0.04	0.03	0.04	0.07
9,11	7.77[†, ac]	8.00 [a]	8.84 [bc]	8.05 [a]	8.34 [abc]	8.29 [abc]	8.08 [abc]	8.88 [b]	0.00
7,9	1.03[†,a]	1.06[ab]	1.14[b]	1.04[a]	1.08[ab]	1.08[ab]	1.05[ab]	1.17[a]	0.00

[†] significant differences by feeding.

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Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization

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ABSTRACT

High pressure homogenization (HPH) is a novel technology that promotes fat globule size reduction and microbial inactivation, but little research exists on the fate of milk fat lipids. This work studied the effect of HPH (0–350 MPa) of raw cow, goat and ewe milks on the fatty acid total content and profile to elucidate whether this technology has a major impact on the lipid fraction of milk and especially on CLA isomers. Fatty acids in processed milks were determined by GC-FID and CLA isomers by Ag+-HPLC.
Our results indicate that the total amount of fat extracted from the milk samples decreased as the homogenization pressure increased, whereas no significant differences were found in the fatty acid composition, especially in the PUFA and CLA isomers profile of raw milk treated by HPH process up to 350 MPa.
Industrial relevance: The absent of significant modifications of the fatty acids content and CLA isomers profile in milk by using high-pressure homogenization is relevant in the development of nonthermal technologies able to pasteurize/sterilize foods, without the organoleptic, functional, and chemical alterations associated to thermal processing.

1. Introduction

Valve homogenization is a standard unit operation in the dairy industry, where fluid milk is forced through a small orifice at pressure ~15 MPa, to reduce the size of fat globules and avoid creaming. High pressure homogenization (also referred as dynamic high pressure) is a similar unit operation but with fluid foods subjected to pressure up to 400 MPa. High pressure homogenization has been used for the stabilization of emulsions and to inactivate harmful bacteria in fluid foods, with minimal organoleptic changes when compared to thermal pasteurization (Hayes, Fox, & Kelly, 2005; Huppertz, Smiddy, Upadhyay, & Kelly, 2006; Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003). This technology differs from high hydrostatic pressure in that relatively lower pressure is applied and that high pressure homogenization is a continuous process where the fluid food is subjected to a combination of pressure, shear stress, cavitation, turbulence and impingement (Shirgaonkar, Lothe, & Pandit, 1998; Floury, Bellettre, Legrand, & Desrumaux, 2004).

The effect of homogenization and high hydrostatic pressure on the components of milk has been widely described. However, little research exists on the use of high pressure homogenization in milk, particularly with regards to its lipid fraction. High pressure homogenization promotes partial whey protein denaturation, casein micelle dissociation, and disruption of the milk fat globule membrane (Hayes et al., 2005; Pereda et al., 2007; Roach & Harte, 2008; Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007). There is also evidence that high pressure homogenization at pressure <100 MPa does not protect milk from enzymatic rancidity due to the lack of inactivation of lipases (Datta, Hayes, Deeth, & Kelly, 2005; Humbert, Driou, Guerin, & Alais, 1980). This lack of inactivation of lipases was also reported by Pereda, Ferragut, Quevedo, Guamis, and Trujillo (2008a), that showed a significant increase in milk FFA only when HPH-treatment was applied to raw milk at 200 MPa and 30 °C but it was not significantly detected at 200 MPa, 40 °C nor 300 MPa at different temperatures.

The effect of processing and storage conditions on the lipid profile of milk is not fully understood and the subject of controversy, particularly for the case of fatty acids with potential health benefits (*e.g.*, anticarcinogenic activity), such as conjugated linoleic acid (CLA) isomers. Steinhart (1996) found that the thermal processing of milk altered the CLA isomer distribution in dairy products while the total CLA content remained unchanged and Lin, Boylston, Luedecke, and Shultz (1999) detected variations in the CLA levels and presence of new isomers in ripened cheeses. Other processing operations (*e.g.*, hydrogenation) were also shown to promote the formation of new CLA intermediate isomers (Jung & Jung, 2002).

Despite numerous studies describing the effect of homogenization in milk and its potential impact in human health (Michalski & Januel,

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2006), there are no reports quantifying individual FA or CLA isomers after high pressure homogenization. Ewe, cow and goat are the main ruminant species used to produce milk for human consumption and it is well known that these milks have different fatty acid profiles (Jensen, 2002). The objective of this research was to determine the effect of high pressure homogenization on the fatty acids relative content of cow, ewe and goat raw milks, with emphasis on CLA isomers.

2. Materials and methods

2.1. Chemicals

Hexane and Methanol were purchased from LabScan (Dublin, Ireland), Potassium hydroxide and Sodium sulphate-1 hydrate from Panreac (Barcelona, Spain), Conjugated linoleic acid standards from Nu-Chek Prep (Elysian, Minnesota, USA), and glyceryl tritridecanoate from Sigma (St. Louis, Missouri, USA). All reagents used in these experiments were GC or HPLC grade. Reference milk fat butter BCR-164 (EU Commissions; Brussels, Belgium) was purchased from Fedelco Inc. (Madrid, Spain).

2.2. Milk processing

Raw whole milk from three different ruminant species (cow, ewe, and goat) was obtained from the University of Tennessee dairy farm and subjected to 50 to 350 MPa homogenization pressure in 50 MPa increments, using a FPG 12500 bench-top high pressure valve homogenizer (Stansted Fluid Power, Ltd., Essex, UK) with inlet temperature of 10°C. In the 50 to 350 MPa pressure range, the temperature of the sample measured after the homogenization valve followed a quadratic regression with respect to pressure ($T=-0.0002*P2+0.2279*P+10$; where T is temperature in °C and P is pressure in MPa). To minimize the thermal effect, samples were rapidly cooled in less than 2 s to 10 °C using a tubular heat exchanger connected immediately after the homogenization valve. After treatment, samples were placed in refrigeration before milk fat extraction. An untreated raw milk sample from each species was used as a control. The experiment was done on duplicate.

2.3. Lipid extraction

For analysis of fatty acid methyl esters (FAME), trans fatty acids (TFA), and CLA isomers, fat from milk samples was extracted by the method proposed by Luna, Juarez, and de la Fuente (2005) based on two centrifugation steps and successfully validated against the official reference procedure (ISO-IDF, 2001) based on solvent extraction, for the determination of the fatty acid profile in ewe's and goat's milk with different fat contents and fat globule size. Briefly, raw milk sample was tempered at 20°C for 20 min, and centrifuged at 17,800×g for 30 min at the same temperature. The fat layer was removed, transferred to microtubes and centrifuged again. Separated lipids were stored in amber vials, exposed to a stream of N2, and frozen at −20°C until analysis.

2.4. Fatty acid determination and quantification

Fatty acid methyl esters (FAMEs) were prepared by base-catalyzed methanolysis of the extracted fatty acid fraction using 2N KOH in methanol as described by International Standard method ISO-IDF (ISO, 2002). FAMEs were analyzed on an Agilent gas chromatography unit (model 6890N, Palo Alto, CA, USA) equipped with a flame ionization detector. Fatty acids were separated using a CP-Sil 88 fused-silica capillary column (100 m×0.25 mm i.d.×0.2 μm film thickness, Chrompack, Middelburg, Netherlands). The column was held at 100°C for 1 min after injection, temperature-programmed at 7°C/min to 170°C, held at 170°C for 55 min, and then temperature-programmed at 10 °C/min to 230°C and held at the final temperature for 33 min. Helium was the carrier gas with a column inlet pressure set at 214 kPa and a split ratio of 1:20. The injector temperature was set at 250 °C and the detector temperature was set at 270 °C. Injection volume was 0.5 μL. Reference butter fat BCR-164 was used to obtain response factors (Rf). For quantitative purposes, tritridecanoin (C13; 12.4 mg/mL) was added to test samples and reference butter fat. The determinations were done on duplicate.

2.5. CLA detection and quantification

A HPLC system equipped with a diode array detector (Shimadzu Vp Series, Duisburg, Germany) was used to detect and quantify individual CLA isomers present in milk fat samples. Absorbance was scanned from 190 to 300 nm wavelength and 233 nm used for isomer quantification. The conjugated linoleic acid methyl esters were separated using a ChromSpher 5 Lipid column (4.6 mm i.d.×250 mm stainless steel; 5 μm particle size; Varian-Chrompack, Middelburg, Netherlands) as described by Rodriguez-Alcalá and Fontecha (2007). Isomers identification was done by comparing the relative retention times from the sample peaks to those of a CLA standard mix, as well as the second derivative of the wavelength spectrum as described by Banni, Day, Evans, Corongiu, and Lombardi (1995).

2.6. Statistical analysis

Data obtained from each chromatographic technique were statistically processed according to one-way ANOVA procedure ($P<0.05$) performed with the aid of the SPSS package (SPSS 15 for Windows, SPSS Inc., Chicago, Illinois, USA).

3. Results and discussion

3.1. Milk fat extraction

The total amount of fat extracted from the milk samples decreased as the homogenization pressure increased from 50 to 350 MPa and milk fat extraction was unfeasible for ewe milk after high pressure homogenization treatment higher than 250 MPa. The drop in extracted fat could be caused by an increase in viscosity of samples as the processing pressure dissociated the fat globule and dispersed the fatty acids throughout the sample, mainly in samples with higher fat content such as ewe's milk. These results are in agreement with those reported by Pereda et al. (2007), where higher viscosity was found after milk was subjected to high pressure homogenization at 300 MPa. These authors found that milks homogenized at pressure ~300 MPa were characterized by the formation of fat aggregates that were not observed at relatively lower homogenization pressure (*e.g.*, ≤200 MPa). Milk is briefly subjected to high hydrostatic pressure when processed using high pressure homogenization and this could potentially lead to difficulties in the recovery of fat by centrifugation. High hydrostatic pressure in the 100–800 MPa range was reported to promote the association between whey proteins (*e.g.*, α-Lactalbumin) and the milk fat globule membrane in whole milk (Ye, Anema, & Singh, 2004). Zulueta, Esteve, Frasquet, and Frigola (2007) also reported similar significantly lower milk fat recovery when milk was subjected to other nonthermal processes (*e.g.*, pulsed electric fields).

3.2. Fatty acid composition

Composition for fatty acids in cow, goat and ewe milks subjected to high pressure homogenization are presented in Tables 1, 2, and 3, respectively. The composition of the major fatty acids as well as their distribution in the saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids fractions were within the range reported elsewhere for cow, ewe and goat milks (Jensen, 2002). Small variations may be due to the known fact that ruminant milk lipid

Table 1
Fatty acid composition (% of total fatty acids) of raw and HPH processed cows milk

Fatty acid	HPH (pressure MPa)								
	0	50	100	150	200	250	300	350	SEM
C4	3.21	3.15	3.15	3.18	3.17	3.17	3.22	3.20	0.091
C6	1.88	1.83	1.82	1.85	1.83	1.86	1.88	1.56	0.032
C8	1.01	0.98	0.97	0.98	0.98	0.99	1.01	0.89	0.013
C10	2.19	2.15	2.13	2.16	2.15	2.18	2.20	1.98	0.027
C10:1	0.23	0.22	0.22	0.23	0.22	0.23	0.23	0.20	0.005
C12	2.39	2.37	2.35	2.37	2.37	2.40	2.41	2.21	0.023
C14	8.21	8.13	8.14	8.17	8.18	8.24	8.22	7.79	0.054
C15i	0.17	0.18	0.19	0.18	0.18	0.18	0.18	0.16	0.004
C15ai	0.34	0.35	0.35	0.35	0.35	0.18	0.35	0.33	0.086
C14:1c	0.60	0.61	0.61	0.62	0.61	0.62	0.62	0.59	0.003
C15	0.75	0.76	0.75	0.75	0.75	0.76	0.75	0.73	0.003
C16i	0.19	0.20	0.20	0.20	0.20	0.20	0.19	0.19	0.004
C16	27.08	27.22	27.22	27.08	27.21	27.24	27.09	27.06	0.144
C17i	0.33	0.32	0.34	0.33	0.33	0.33	0.31	0.32	0.010
C17ai	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.026
C16:1 c	1.37	1.37	1.38	1.40	1.40	1.39	1.39	1.47	0.047
C17	0.44	0.45	0.44	0.44	0.44	0.44	0.43	0.46	0.015
C17:1c	0.14	0.14	0.13	0.14	0.14	0.14	0.13	0.14	0.007
C18	11.66	11.83	11.76	11.61	11.72	11.64	11.50	11.68	0.500
18:1 4t	0.06	0.05	0.06	0.06	0.06	0.05	0.06	0.05	0.009
C18:1 5t	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.005
C18:1 6-8t	0.46	0.46	0.46	0.46	0.46	0.46	0.47	0.59	0.021
C18:1 9t	0.40	0.39	0.39	0.38	0.38	0.38	0.38	0.39	0.007
C18:1 10t	0.68	0.74	0.71	0.69	0.73	0.74	0.70	0.71	0.038
C18:1 11t VA	1.73	1.69	1.71	1.73	1.70	1.68	1.70	1.71	0.039
C18:1 12-13t+7c	0.62	0.65	0.63	0.61	0.56	0.62	0.67	0.62	0.035
C18:1 c9+15t	23.70	23.56	23.65	23.78	23.72	23.69	23.75	23.69	0.199
C18:1 11c	1.15	1.14	1.14	1.14	1.14	1.14	1.14	1.22	0.014
C18:1 12c	0.70	0.68	0.70	0.70	0.68	0.70	0.70	0.74	0.018
C18:1 13c	0.08	0.08	0.08	0.05	0.07	0.08	0.06	0.34	0.131
C18:1 14c+16t	0.59	0.59	0.59	0.59	0.60	0.59	0.58	0.38	0.100
C18:1 15c	0.15	0.16	0.17	0.18	0.16	0.17	0.18	0.12	0.029
C18:2 t,t NMID	0.39	0.39	0.39	0.38	0.39	0.39	0.39	0.31	0.036
C18:2 t9,t12	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.037
C18:2 c9,t13	0.24	0.24	0.25	0.25	0.25	0.25	0.25	0.19	0.023
C18:2 c9,t12	0.09	0.10	0.10	0.09	0.10	0.09	0.09	0.07	0.006
C18:2 11t,15c	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.031
C18:2 9t,12c	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.04	0.029
C18:2 c9,c12 n6	3.62	3.59	3.60	3.61	3.61	3.62	3.64	3.61	0.364
C20	0.19	0.19	0.19	0.19	0.19	0.20	0.18	0.15	0.019
αC18:3 n3	0.31	0.32	0.32	0.32	0.31	0.31	0.32	0.33	0.017
C18:2 c9,t11 CLA	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.005
C22	0.04	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.001
C20:3 n6	0.12	0.11	0.12	0.12	0.12	0.11	0.11	0.12	0.002
C20:4 n6	0.13	0.12	0.12	0.13	0.12	0.12	0.12	0.13	0.002
C20:5 n3	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.002
C22:5 n3	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.003
Σ SFA	60.51	60.58	60.47	60.31	60.51	60.47	60.39	60.22	0.334
Σ MUFA	32.86	32.74	32.87	33.00	32.86	32.91	32.98	32.89	0.248
Σ PUFA	5.72	5.71	5.72	5.72	5.71	5.71	5.74	5.72	0.463
Total *trans*	4.54	4.57	4.55	4.53	4.48	4.53	4.57	4.02	0.079
Total CLA	0.56	0.56	0.56	0.56	0.56	0.55	0.55	0.54	0.012

Values represent mean of duplicate analyses of two individual samples.
Different superscripts refer to significant variations by HPH processing ($P<0.05$).
Abbreviation: *trans* (t), *cis* (c), iso (i), anteiso (ai), Vaccenic acid (VA), conjugated linoleic acid (CLA), Non methylene interrupted diene (NMID).

composition is affected by genetic (*e.g.*, breed) and environmental factors (*e.g.*, diet, management) (Bauman & Griinari, 2003).

As expected, the most prevalent fatty acid fraction corresponded to SFA with 60, 66 and 64% relative content for cow, goat and ewe milks, respectively. The main fatty acids in the SFA fraction were Palmitic acid (C16:0) followed by Stearic acid (C18:0) for all samples, independently of the species or homogenization treatment. Slight variations among the various fatty acid fractions were found during the study but were not statistically significant and therefore not related to the processing by high pressure homogenization. Within the unsaturated fatty acid fraction, the major fatty acid present in quantitative terms was Oleic acid (C18:1 *cis*9) with relative concentration of 24, 20 and 20% for cow, goat and ewe milks, respectively. Palmitoleic acid (C16:1 *cis*9) was prevalent among *cis* MUFA, with 1.4% for cow milk and 1% for goat and ewe milks. Also C18:1*cis*11 was present in 1.1, 0.7 and 0.8% relative content for cow, goat and ewe milks, respectively. In all cases, statistical analysis yielded no significant differences in fatty acid composition as affected by high pressure homogenization processing. The total *trans* MUFA concentration in samples was 4.5, 4.3 and 5.3% for cow, goat, and ewe milks, respectively. Vaccenic acid (C18:1 *trans*11) was the prevalent *trans* MUFA fraction, with average contents of 1.7, 2.1 and 2.8% of total fatty acid, respectively. Since Vaccenic acid is a precursor of the major CLA isomer C18:2 *cis*9*trans*11 (Rumenic acid) in the mammary gland of ruminants and humans (Turpeinen et al., 2002), it is important that the processing operations such as high pressure homogenization do not reduce its relative content in milk. The C18:1 *trans*10 moiety was present in 0.7% for cow and ewe milks and 0.5% for goat milk regardless of the homogenization treatment.

Table 2
Fatty acid composition (% of total fatty acids) of raw and HPH processed goats milk

Fatty acid	HPH (pressure MPa)								
	0	50	100	150	200	250	300	350	SEM
C4	2.26	2.30	2.32	2.31	2.33	2.38	2.41	2.48	0.038
C6	2.55	2.50	2.53	2.53	2.56	2.63	2.65	2.72	0.036
C8	2.62	2.56	2.57	2.64	2.66	2.73	2.74	2.80	0.034
C9	0.15	0.14	0.14	0.15	0.15	0.15	0.15	0.16	0.002
C10	8.61	8.41	8.40	8.74	8.75	8.93	8.90	9.05	0.074
C10:1	0.37	0.36	0.35	0.38	0.38	0.39	0.38	0.39	0.004
C12	3.29	3.27	3.23	3.38	3.35	3.39	3.34	3.32	0.016
C14i	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.13	0.003
C14	7.07	7.12	7.07	7.14	7.08	7.07	7.01	6.98	0.039
C15i	0.26	0.26	0.26	0.27	0.27	0.27	0.27	0.27	0.014
C15ai	0.38	0.39	0.39	0.39	0.40	0.39	0.40	0.42	0.004
C14:1c	0.10	0.11	0.11	0.12	0.12	0.12	0.12	0.13	0.007
C15	1.23	1.23	1.22	1.22	1.22	1.21	1.20	1.22	0.008
C16i	0.25	0.25	0.25	0.25	0.14	0.24	0.25	0.25	0.060
C16	25.95	26.14	26.01	25.51	25.38	25.05	25.02	25.36	0.134
C17i	0.47	0.47	0.47	0.47	0.46	0.46	0.45	0.47	0.009
C17ai	0.39	0.39	0.40	0.39	0.40	0.41	0.42	0.44	0.002
C16:1 c	1.06	1.08	1.07	1.07	1.09	1.10	1.10	1.14	0.011
C17	0.77	0.78	0.76	0.76	0.75	0.73	0.73	0.73	0.006
C17:1c	0.29	0.29	0.29	0.28	0.29	0.30	0.33	0.31	0.010
C18	9.45	9.61	9.61	9.23	8.97	8.69	8.61	8.47	0.083
C18:1 6-8t	0.37	0.38	0.38	0.37	0.38	0.38	0.37	0.40	0.010
C18:1 9t	0.40	0.39	0.39	0.40	0.39	0.39	0.40	0.43	0.007
C18:1 10t	0.53	0.58	0.58	0.56	0.56	0.52	0.55	0.63	0.038
C18:1 11t VA	2.15	2.11	2.13	2.10	2.12	2.13	2.12	2.08	0.032
C18:1 12t	0.45	0.46	0.46	0.43	0.42	0.43	0.42	0.59	0.032
C18:1 c9+15t	19.91	19.80	20.08	20.24	20.64	20.85	20.16	20.02	0.064
C18:1 11c	0.74	0.73	0.74	0.72	0.73	0.73	0.75	0.75	0.021
C18:1 12c	0.24	0.24	0.23	0.24	0.23	0.23	0.24	0.28	0.009
C18:1 13c	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.08	0.006
C18:1 14c+16t	0.37	0.37	0.37	0.37	0.36	0.36	0.35	0.38	0.007
C18:1 15c	0.11	0.12	0.12	0.12	0.12	0.11	0.11	0.13	0.003
C18:2 t,t NMID	0.24	0.29	0.29	0.30	0.30	0.31	0.31	0.35	0.026
C18:2 c9,t13	0.20	0.16	0.21	0.21	0.21	0.17	0.04	0.28	0.036
C18:2 c9,t12	0.06	0.05	0.06	0.05	0.05	0.05	0.05	0.11	0.056
C18:2 11t,15c	0.04	0.04	0.04	0.05	0.04	0.04	0.04	n.d.	0.002
C18:2 9t,12c	0.09	0.08	0.09	0.09	0.08	0.09	0.09	0.10	0.009
C18:2 c9,c12 n6	1.98	1.96	1.96	2.01	2.05	2.08	2.08	2.09	0.013
C18:2 c9,c15	0.11	0.11	0.14	0.12	0.14	0.14	0.14	0.13	0.091
C20	0.45	0.45	0.44	0.43	0.41	0.40	0.21	0.38	0.006
αC18:3 n3	0.42	0.42	0.43	0.42	0.43	0.45	0.44	0.45	0.014
C18:2 c9,t11 CLA	0.63	0.64	0.64	0.65	0.66	0.67	0.65	0.69	0.023
C20:3 n6	0.02	0.03	0.02	0.02	0.02	0.02	0.02	n.d.	0.002
C20:4 n6	0.11	0.11	0.11	0.12	0.12	0.12	0.12	0.13	0.023
C20:5 n3	0.03	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.003
C22:5 n3	0.01	0.02	0.02	0.02	0.02	0.01	0.01	n.d.	0.013
Σ SFA	66.55	66.79	66.57	66.30	65.78	65.65	65.22	65.89	0.102
Σ MUFA	27.40	27.28	27.58	27.66	28.10	28.30	28.66	28.30	0.082
Σ PUFA	4.08	4.14	4.18	4.25	4.31	4.34	4.37	4.43	0.086
Total *trans*	4.35	4.38	4.39	4.31	4.30	4.30	4.47	4.48	0.093
Total CLA	0.71	0.72	0.72	0.73	0.74	0.76	0.77	0.69	0.014

Values represent means of duplicate analyses of two individual samples.
Different superscripts refer to significant variations by HPH processing ($P<0.05$).
Abbreviation: *trans* (t), *cis* (c), iso (i), anteiso (ai), Vaccenic acid (VA), conjugated linoleic acid (CLA), Non methylene interrupted diene (NMID).

Table 3
Fatty acid composition (% of total fatty acids) of raw and HPH processed ewes milk

Fatty acids	HPH (pressure MPa)						
	0	50	100	150	200	250	SEM
C4	3.60	3.56	3.58	3.68	3.65	3.68	0.025
C6	2.78	2.55	2.93	2.76	2.66	2.71	0.143
C8	2.46	2.13	2.50	2.36	2.27	2.31	0.109
C10	7.00	6.24	6.81	6.71	6.50	6.57	0.151
C10:1	0.30	0.25	0.28	0.28	0.28	0.28	0.003
C12	3.84	3.52	3.79	3.73	3.61	3.64	0.065
C14i	0.13	0.13	0.13	0.14	0.13	0.14	0.002
C14	8.57	8.45	8.57	8.65	8.60	8.57	0.034
C15i	0.23	0.24	0.23	0.24	0.24	0.24	0.004
C15ai	0.51	0.52	0.51	0.53	0.53	0.52	0.007
C14:1c	0.12	0.13	0.13	0.14	0.13	0.13	0.006
C15	0.97	0.99	0.98	0.99	0.98	0.98	0.007
C16i	0.32	0.32	0.31	0.32	0.31	0.31	0.006
C16	20.86	21.86	21.24	21.26	21.29	21.33	0.189
C17i	0.60	0.62	0.60	0.60	0.60	0.60	0.003
C17ai	0.40	0.40	0.39	0.41	0.40	0.41	0.003
C16:1 c	0.97	0.99	0.97	1.00	1.00	1.00	0.005
C17	0.61	0.65	0.60	0.60	0.60	0.59	0.008
C17:1c	0.20	0.19	0.20	0.20	0.19	0.19	0.007
C18	10.65	11.58	10.81	10.47	10.89	10.56	0.102
C18:1 6-8t	0.34	0.34	0.34	0.34	0.31	0.34	0.016
C18:1 9t	0.33	0.32	0.32	0.34	0.33	0.33	0.007
C18:1 10t	0.74	0.77	0.71	0.83	0.69	0.73	0.051
C18:1 11t VA	2.93	2.92	2.90	2.73	2.91	2.89	0.050
C18:1 12t	0.45	0.44	0.43	0.52	0.45	0.45	0.019
C18:1 c9+15t	20.03	20.10	19.96	20.23	20.43	20.51	0.119
C18:1 11c	0.85	0.87	0.85	0.84	0.85	0.85	0.007
C18:1 12c	0.32	0.32	0.31	0.31	0.30	0.31	0.011
C18:1 14c+16t	0.58	0.58	0.57	0.55	0.56	0.55	0.006
C18:1 15c	0.17	0.17	0.16	0.17	0.16	0.16	0.010
C18:2 t,t	0.38	0.37	0.37	0.38	0.37	0.38	0.008
C18:2 c9,t13	0.22	0.22	0.22	0.23	0.22	0.22	0.004
C18:2 c9,t12 n6	0.08	0.08	0.08	0.08	0.07	0.07	0.003
C18:2 11t,15c	0.04	0.04	0.04	0.04	0.04	0.04	0.004
C18:2 9t,12c	0.18	0.18	0.18	0.18	0.18	0.18	0.004
C18:2 c9,c12	3.05	2.96	2.98	3.03	3.03	3.06	0.016
C18:2 c9,c15	0.06	0.06	0.06	0.07	0.07	0.07	0.006
C20	0.26	0.29	0.27	0.27	0.15	0.25	0.067
αC18:3 n3	0.50	0.48	0.49	0.66	0.66	0.50	0.128
C18:2 c9,t11 CLA	0.87	0.85	0.85	0.88	0.87	0.88	0.001
C22	0.08	0.08	0.07	0.08	0.07	0.08	0.002
C20:3 n6	0.03	0.02	0.02	0.03	0.05	0.03	0.015
C20:4 n6	0.13	0.12	0.13	0.13	0.07	0.13	0.032
C20:5 n3	0.04	0.04	0.04	0.04	0.08	0.04	0.026
C22:5 n3	0.02	0.02	0.02	0.03	0.03	0.02	0.004
Σ SFA	64.12	64.27	64.36	64.24	64.57	64.55	0.285
Σ MUFA	28.67	28.67	28.73	28.81	28.49	28.48	0.203
Σ PUFA	5.82	5.73	5.66	5.70	5.68	5.74	0.118
Total *trans*	5.46	5.45	5.43	5.41	5.31	5.33	0.063
Total CLA	1.00	0.99	0.97	0.98	0.98	0.99	0.006

Values represent means of duplicate analyses of two individual samples.
Different Superscripts refer to significant variations by HPH processing ($P<0.05$).
Abbreviation: *trans* (t), *cis* (c), iso (i), anteiso (ai), Vaccenic acid (VA), conjugated linoleic acid (CLA), Non methylene interrupted diene (NMID).

The total relative content of PUFA fraction was 5.7, 4.4, and 5.8% for cow, goat and ewe milks, respectively and it was mainly composed by Linoleic acid (C18:2 *cis*9*cis*12) with 3.6, 2 and 3% for cow, goat, and ewe milks and Linolenic acid (C18:3 *cis*9*cis*12*cis*15) with 0.3, 0.4, and 0.6% for cow, goat and ewe milks. Rumenic acid (C18:2 *cis*9*trans*11) was the main moiety (0.6, 0.7 and 1.0% for cow, goat and ewe milks, respectively) within total CLA content. The CLA values obtained by GC chromatography were well supported by prior research reports (Bisig, Eberhard, Collomb, & Rehberger, 2007), and agree with the identity of the greater isomers as well as the observed concentrations.

3.3. CLA isomer profile

Results obtained from the Ag+-HPLC separation of CLA isomers in both control and high pressure homogenized samples are shown in Table 4. Although the CLA content in milk and dairy products is affected by many factors such as raw material production, processing, aging and storage (Khanal, 2004), the values obtained in this work were in the same range as those previously reported elsewhere for native cow, ewe, and goat milks (Calvo, Kives, Romero, & Fontecha, 2007; Jensen, 2002; Luna, Fontecha, Juarez, & de la Fuente, 2005).

As expected, in all studied samples the major positional isomer determined was the C18:2 *cis*9–*trans*11 (Rumenic acid), with relative content of 81–85% of total CLA. The C18:2 *cis*7–*trans*9 was the second most relevant CLA, accounting for 10, 5, and 8% of total CLA for cow, goat and ewe milks, respectively. No significant alteration either in quantity or distribution of these major CLA isomers was observed as a result of the processing of milk using high pressure homogenization up to 350 MPa. The total amount of the remaining minor isomers found in the studied samples was less than 10% of the total CLA fraction. Nevertheless, some of the minor CLA isomers are important due to their biological activities such as C18:2 *trans*10–*cis*12, responsible for observed weight loss and muscle-mass enhancement effects (Park & Pariza, 2007). Whereas this fraction is almost not detected in milk samples, it is prevalent in commercial CLA preparations produced by alkaline isomerization of linoleic acid. The content of the C18:2 *cis*11–*trans*13 isomer in the studied samples was found to be 4-fold higher in goat than in cow milk. The total content of trans–trans isomers found in the present study was between 5.5 and 8.5% of total CLA. Neither of these compounds displayed significant variations attributable to HPH processing.

The HPH technology permits the production of milk fine emulsions because the fat globule size decreased as HPH pressure increased (Hayes et al., 2005), but little published data is available regarding modification of lipid constituents as PUFA and CLA contents. Some authors have reported an increase of lipolysis in milk, when HPH-treatment was applied to raw milk under certain conditions of pressure and temperature (Hayes & Kelly, 2003, Datta et al., 2005; Pereda et al., 2008a). They suggested that lipolysis in milk can be produced by the action of natural lipases not completely inactivated by the conditions of pressure–temperature used in their studies. Pereda et al. (2008a) reported a significant increase of FFA when HPH-treatment was applied to raw milk only at 200 MPa 30 °C but not at 200 MPa 40 °C nor 300 MPa. Nevertheless, the type and quality of raw materials, besides of the processing conditions, seem to greatly influence the enzyme action and therefore the free radicals and accumulation of oxidation compounds. Thus, same authors (Pereda, Jaramillo, Quevedo, Ferragut, Guamis, & Trujillo, 2008b) found much less production of volatile compounds in HPH treated samples compared to heat treated commercial milk samples and concluded

Table 4
CLA isomers composition (% isomer of total CLA) of raw and HPH processed milk samples analysed by Ag^+-HPLC

CLA isomers	Cows milk			Goats milk			Ewes milk		
	Raw	350 MPa	SEM	Raw	350 MPa	SEM	0	250 MPa	SEM
Total *t*–*t*	7.61	7.25	0.468	8.49	7.43	0.939	5.62	5.71	0.264
*t*12–*t*14	0.49	0.50	0.120	0.80	0.78	0.101	1.03	1.09	0.285
*t*11–*t*13	0.88	0.84	0.067	0.75	0.66	0.089	1.04	1.01	0.096
*t*10–*t*12	1.68	1.90	0.337	2.56	2.36	0.189	0.98	0.93	0.050
*t*9–*t*11	1.90	1.54	0.200	1.51	1.30	0.191	0.81	0.85	0.050
*t*8–*t*10	1.03	1.08	0.147	1.89	1.51	0.190	1.08	1.10	0.070
*t*7–*t*9	1.10	0.93	0.085	0.36	0.31	0.077	0.69	0.72	0.050
*t*6–*t*8	0.53	0.45	0.057	0.61	0.52	0.108	n.d.	n.d.	–
Total *c*,*t*–*t*,*c*	92.39	92.75	0.468	91.51	92.57	0.939	94.38	94.29	0.264
*t*11–*c*13	0.47	0.42	0.012	1.66	1.75	0.011	0.86	0.76	0.072
*c*9–*t*11	81.86	82.36	0.146	84.96	86.07	0.880	85.53	85.92	0.185
*c*7–*t*9	10.06	9.97	0.333	4.89	4.75	0.074	7.98	7.61	0.343

Values represent means of duplicate analyses of two individual samples.
Different superscripts refer to significant variations by HPH processing ($P<0.05$).
Abbreviation: *trans* (t), *cis* (c).

that HPH process seems to be a good alternative to traditional heat treatments.

On the other hand, Lynch et al. (2005) studying the thermal pasteurization of milk, did not find any effect of thermal pasteurization over the lipid profile of milks with naturally enhanced CLA and Vaccenic acid contents, similarly to other authors who focused in CLA and trans fatty acids (Herzallah, Humeid, & Al-Ismail, 2005). Vazquez-Landaverde, Torres, and Qian (2006) also studied the effect of thermal processing together with high hydrostatic pressure processing on the formation of volatile compounds in cow's milk (*e.g.*, short chain fatty acids), and concluded that no differences were observed.

4. Conclusions

Our results indicate that the HPH in the 50–350 MPa pressure range does not significantly impact the fatty acid content and CLA isomers profile in cow, ewe and goat milks. Therefore, high pressure homogenization is a potential technology for the pasteurization of the ruminant milk tested with minimal changes in the fatty acids total content.

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Cow milk processed at very high pressure: effects on the fatty acids and phospholipids composition.

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ABSTRACT:

Two cow milk batches, collected in April and June, were processed by very high pressures in a range of 250-900 MPa to study the possible effects of processing in the fatty acid and phospholipids composition. Milk collected in April had lower SFA and higher MUFA and PUFA concentrations as well as in PE, PI, PS and PC ($p<0.05$).

No changes were observed in the fatty acids in none of the pressures levels used. Instead phosphatidylserine and phosphatidylinositol showed lower contents ($p<0.05$) after all the pressures as MFGM is being disrupted by the inclusion of proteins as result of processing by high pressures and the extension of the alteration was affected by the composition.

1. Introduction:

In the processing of dairy products is a common step the use of heating procedures as pasteurization, sterilization or UHT, to assure the absence of harmful microorganism for the consumer, instead they can alter sensory characteristics of the food (Smiddy, Martin, Huppertz &, Kelly, 2007).

The presence of polyunsaturated fatty acids (PUFA) in milk, as well as an increasing tendency in the dairy industry to enhance its contents against saturated (Parodi, 2004) and their susceptibility to oxidative changes and alterations due to heating processing (Campbell, Drake &, Larick, 2003, Rodriguez-Alcala &, Fontecha, 2007), drives to search and develop less aggressive methods for organoleptic and physicochemical properties.

Recently, phospholipids have gain attention due to exhibited healthy properties as agents against colon cancer, gastrointestinal pathogens, Alzheimer's disease, depression, and stress (Fong, Norris &, MacGibbon, 2007, German &, Dillard, 2006, Spitsberg, 2005). Cow milk has a lipid content of 3-5% where phospholipds represent 0.1 to 1% (Sánchez-Juanes, Alonso, Zancada &, Hueso, 2009). They are major constituents of milk fat globule membrane (60-90%): it is arranged as a trilayer structure where the primary one (inner, in contact with tryglicerides) comes from the endopolasmatic reticulum membrane as a result from the liberation of the fat globule to the cytoplasm of mammary gland cell. The others two are from the apical plasma membrane when it is secreted into milk (Lopez, Briard-Bion, Menard, Rousseau, Pradel &, Besle, 2008). Phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidyliserine are located in the inner layer whilst phosphatidylcholine and sphingomyelines are in the outer.

High-pressures joins in a same process antimicrobiolical properties of heating treatments, at the same time does not change sensory characteristics (Huppertz, Smiddy, Upadhyay &, Kelly, 2006, Lopez-Fandino, 2006).
However, apparition of stress forces (cavitation, friction, turbulence and shear), due to whey and casein protein denaturalization, while the milk fat globule membrane is disrupted, acts on the food functional properties. The MFGM composition was altered

when recomposing so that denaturized proteins can now be integrated into it (Michalski &, Januel, 2006, Patazca, Koutchma &, Balasubramaniam, 2007, Zamora, Ferragut, Jaramillo, Guamis &, Trujillo, 2007).

Current scientific bibliography is vast describing the high pressure effects in milk proteins but scant in lipids and fats behaviour. It opens a field to be studied in view of evidences pointing out toward high pressures processing can cause globule fat reduction and milk native lipase noninactivation, increasing the risk of lipolysis (Datta, Hayes, Deeth &, Kelly, 2005, Hayes, Fox &, Kelly, 2005). Othe investigations reports odd flavours ascribed to oxidation when food is processed at 40-60 MPa (Humbert et al in Michalski et al, 2006 (Michalski, et al., 2006).

The aim of this study is to obtain results showing the possible effect of high pressures (250-900 MPa) in the fraction of fatty acids and polar lipids of milk.

2. Methods and Materials:

2.1. Samples

The milk batches were used in this study, supplied by a Spanish dairy company and collected in April (1A) and June (2A). Milks were processed by high pressure pilot equipment made by NCHyperbaric, in the facilities of IRTA-CENTA (by duplicate), and sent to our laboratory at the Instituto del Frío in cooling conditions: 7 batches, 6 and a control were subjected to high pressure treatment at different working conditions as shown in Table 1. In order to avoid changes in transportation, a preservative was added (sodium azide) and samples were storage in refrigeration until analysis (within 24 hours).

2.2. Chemicals

All the organic solvents in the present work (Chlroform, Metanhol and Trimethylpentane) were HPLC grade, purchased to LABSCAN (Dublin, Ireland). Triethylamine (99.5%) and Formic acid (98%) were purchased to Sigma (Bellefonte, PA, USA). Cholesterol, cholesterol ester, tritridecanoin, monoacylglicerol, dioleinglicerol, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphigomieline (SP) were also obtained from Sigma. For quantitative determinations of total fatty acid methylesters (FAME), an anhydrous milk fat (reference material BCR-164; EU Commission; Brussels, Belgium, purchased from Fedelco Inc., Madrid, Spain) was used. An internal standard (12.4 mg/mL of C13:0 as triacylglyceride; (Sigma, St. Louis, MO) was also used.

2.3. Samples and Extraction

The isolation of the lipid fraction was carried out within 24 hours since reception, by duplicate and according to the Folch method (1957) to ensure the completed extraction of the whole lipid classes; briefly: 4 g of samples was dissolved in distilled water, 75 mL Chloroform/Metanhol (2:1) was added and vortexed 2 minutes and then centrifuged 5 minutes at 5000 rpm; the lower layer (containing the lipids) was recovered and process repeated but adding 50 mL of Chloroform. Organic solvent centrifuged (5

minutes, 5000 rpm) to avoid the presence of impurities and solvent was removed in a rotary evaporator Samples stored in amber vials at -20ºC until analysis.

2.4. Analysis of the fatty acids

For the determination of the fatty acid composition, samples were derivatized in their methyl esters (FAME) by methoxylation with 2N methanolic KOH solution using the reference procedure of the ISO-IDF (ISO, 2002). The FAME were analyzed according Fontecha et al., (2000), in a gas chromatograph-mass Agilent mod. 6890, using a CP-Sil88 100m column (Varian) with an internal diameter of 0.25 mm (Chrompack, Middelburg, Netherlands), helium as carrier gas (30 psig) and a split ratio of 1 / 20. The temperature program were as follows: 100 º C, 1 minute, ramp 7 ° C / min up to 170 º C, keeping 55 minutes, ramp 10 ° C / min to 230 º C for 33 minutes and maintenance. Injector temperature: 250 º C, detector (FID): 270 º C. The injection volume was 0.5 mL.

2.5. Analysis of polar lipids by HPLC-ELSD

Separation of polar lipids was carried out in a HPLC (Shimadzu Vp Series, Duisburg, F.R. Germany) coupled with an ELSD detector (SEDERE. SEDEX 85 model, Alfortville Cedex, FRANCE) using filtered air as the nebulizing gas at a pressure of 3.5 bar, at 60 ºC and the gain was set at 3. A 250mm x 4.5 mm Zorvax Rx-SIL column (Agilent Tecnhnologies, Palo Alto, CA, USA) with 5-μm particle diameter was used.
The elution program was a linear gradient with A: 87.5: 12:0.5(vol/vol/vol) Chloroform:Methanol:Formic acid (1 *M,* pH 3 adjusted with triethylamine*);* B: 28/60/12 of B. The flow rate was 1 mL/min, the injection volume was 25 μL and the column was equilibrated at 40ºC. Injections were performed at least by duplicate and in different days with a fresh preparation of samples and buffers.
Retention times of the compounds of interest were tested by injection of internal standards in proper concentrations.

Samples were prepared in a concentration of 0.5 mg / mL (Chloroform). The identification of individual polar lipids (phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and sphigomieline) was conducted by

comparing the retention times of pure reference compounds. The calibration of the detector was performed using regression curves that related the area against the amount injected of each compound (15-2.5µg) in duplicate, yielding a good correlation (R^2>0.99) in all cases.

2.4. Samples and Extraction

Full factorial data analysis (one way Anova and GLM; $p<0.05$) was conducted with the aid of the SPSS package (SPSS 17.0 for Windows, SPSS Inc.).

3. Results and discussion:

3.1. Effect in the fatty acid composition

The fatty acid composition of samples from both assays (0-900 MPa) is presented in Tables 2a and 2b. In all cases the major fraction corresponded with that of SFA (saturated fatty acids), where the most relevant compounds were palmitic (C16), stearic (C18) and myristic (C12). In the total of monounsaturated compounds (MUFAs), were oleic (C18:1 9c), palmitoleic (C16:1), vaccenic as well as C18:1 11c in samples in the second trial. The group of polyunsaturated fatty acids (PUFAs) had the lower content; the main compounds according to its concentration were linoleic, linolenic, and rumenic.

The detailed distribution coincides with those reviewed elsewhere (Jensen, 2002). On the other hand the contents among milks from both experiences differ in the amounts of the fatty acids ($p<0.05$): SFAs showed higher concentrations of myristic, palmitic and stearic acids ($p<0.05$) in the samples processed in the second experience (2A) and furthermore the relative contents of MUFAs and PUFAs were lower ($p<0.05$) as the total contents as well as those for C18:1 *trans* 10, oleic and linoleic acid. Rumenic acid had a 1.4-fold increase.

As, explained in the methods and materials section, milks were collected with a difference of three months: genetics (breed and selection), stage of lactation, mastitis,

196 and feed factors have been described as affecting the milk fat composition (Bauman &,
197 Griinari, 2003, Chilliard, Glasser, Ferlay, Bernard, Rouel &, Doreau, 2007).
198
199 Have been described that pasteurization of milk increases the concentration of dissolved
200 oxygen, some proteins can act as hydrogen donors when denatured and some Maillard
201 compounds exert antioxidative properties than UHT (Giroux, St-Amant, Fustier,
202 Chapuzet &, Britten, 2008). Then, instead of could be thought, low temperature
203 processing causes oxidized conditions that it can lead to alterations of the fatty acids
204 profiles. Elsewhere have been reported that pasteurized milk showed higher contents of
205 total trans fatty acids and CLA (Herzallah, Humeid &, Al-Ismail, 2005), which
206 according to Precht et al.(Precht, Molkentin &, Vahlendieck, 1999), is an effect of
207 isomerization of Oleic acid into elaidic (C18:1 9t) due to oxidative reactions and
208 linoleic towards conjugated linoleic acid isomers (Destaillats &, Angers, 2005). Other
209 authors reported than naturally enriched CLA pasteurized milk showed losses in the
210 amounts of rumenic acid (Campbell, et al., 2003) and increments in the *trans,trans* CLA
211 isomers in commercial powdered milk obtained from skim milk added with high CLA
212 content milk. On the other hand, there are thermoresistant lipases not inactivated by
213 heating leading to lower contents of SFA, oleic and linoleic even in UHT milk (Jantova,
214 Vorlova &, Drackova, 2006, Liu, Holland &, Crow, 2004, Panfil-Kuncewicz,
215 Kuncewicz &, Juskiewicz, 2005).
216
217 The available bibliography about possible effects of high pressure processing of milk on
218 the fatty acids is scarce. Serra et al, (Serra, Trujillo, Pereda, Guamis &, Ferragut, 2008)
219 in milk processed by ultra high pressure homogenization found that in general the
220 composition of the free fatty acids along the conservation period was not changed but
221 when conditions were 200MPa at the end of storage the concentrations of stearic , oleic
222 and linoleic acids increase as result of intracellular stearases. Other experiences also
223 reported increasing levels of FFA in milk processed at 200 MPa but no changes in the
224 oxidation parameters in the storage period.
225
226 The comparison of the distribution and fatty acid content in cow milk samples treated at
227 different pressures (0-900MPa) shows no variations related to processing ($p<0.05$).
228 Previous expericences carried out in high-pressure homogenization (0-350 MPa) in
229 cow, goat and sheep milk did no found changes in the fatty acid composition

attributable to processing (Rodriguez-Alcala, et al., 2007). These results are consistent with previous research elsewhere (Huppertz, et al., 2006, Prestamo &, Fontecha, 2007, Rodríguez-Alcala, Harte &, Fontecha, 2008) and agree with those reported in the present research work.

3.2. Effect in the phospholipids composition

The HPLC-ELSD analysis performed shows that in all samples the main components were phosphatidylethanolamine, phosphatidylcholine and sphingomyelines (Table 3a, 3b). As in the fatty acid distribution differences in the contents of phospholipids ($p<0.05$) were found between both groups of samples. Milks 1A had higher concentrations of PE, PC, PI and PS, while SP were lower than milks 2A what elsewhere had been reported as effects of genetics, feeding and state of lactation (Chilliard, et al., 2007, Lopez, et al., 2008). The distributions found for this fraction correspond to those of other authors previously published (Avalli &, Contarini, 2005, Jensen, 2002, Rombaut, Dewettinck &, Van Camp, 2007).

The possible effects of processing in the phospholipids fraction have not been extensively studied as other milk compounds. Current blbliography is scarce.

Some authors point out that thermal processing lead to interactions among whey proteins and the milk fat globule membrane altering the composition of phospholipids: in pasteurized buttermilk was found lower contents of PE, PI and PS as well as in the total content of phospholipids when powder buttermilk is obtained which is explained according to the fact that MFGM in buttermilk is not globular as in milk but laminar and components are exposed against possible interactions (Morin, Jimenez-Flores &, Pouliot, 2007).

This is the first study using high pressures in milk and focused in the effects in fatty and phospholipids composition. The results shows high pressure processing caused decreases ($p<0.05$) of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine concentrations in the range among 250-800 MPa in the first assay (1A) while in the second assay (2A) only PI and PS showed losses at all processing pressures ($p<0.05$) when compared to raw milk. Concentrations of phosphatidylethanolamine significantly increased ($p<0.05$) as response to the decreases

of phosphatidylinositol and phosphatidylserine. Equally, phosphatidylcholine and sphigomyelines had higher concentration ($p>0,05$) as a compensatory effect of the lower levels of PE, PI and PS in the 1A samples. The results points out that PI and PS are prone compounds to alteration and that effects in the phospholipids fraction are distinctively affected by composition.

These three compounds (PE, PS and PI) are located in a inner side of the external bilayer of the fat globule membrane, while in the outer side are phosphatidylcholine and sphingomyelines. Elsewhere has been reported that all the processing steps (handling, agitation, presence of gas bubbles) as well storage of dairy products, can alter the MFGM, due to the inclusion in the fat globule of proteins and its interaction with the compounds of the membranes (Evers, 2004, Michalski, 2007). Other authors have suggested that these alterations are carried out by the action of phospholipases, releasing the fatty acids of phospholipids (Christie, 1982, Rombaut, et al., 2007).

The effects observed in the phospholipids fraction according to very high pressures processing where concentrations PE, PI and PS where decreased in the 1A and in 2A, with the exception of PE for this latter group of samples, may be explained as the inclusion of proteins in the MFMG that finally react with phospholipids located in the inner side of the membrane. Furthermore these alterations were affected at once by the phospholipids composition what points out to the disruption of the MFGM is taken place on a different way.

4. Conclusion

The outcome of present research is that high pressure processing of milk in a range from 250 to 900 MPa, have no effect in its the fatty acid composition but cause losses in the concentrations of PE, PI and PS due to disruption of the MFGM by proteins, then being possible the action of these proteins and phospholipases. The distinctive effect on the reduction in the concentration of PE, point out to a link with the composition of milk.

This is first research work studying the effects of very high pressures on the composition of fatty acids and phospholipids of milk. Further investigations must be focused to know if proteins, phospholipases or a combination of both are the altering agent as well as the mechanism involved.

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299

300 **Table 2a. Fatty acid composition (g FA/100 g fat) in raw milk and processed by high pressure (250-**
301 **900)**; **1st assay (1A).**

	Raw	250 MPa	450 MPa	550 MPa	700 MPa	800 MPa	900 MPa	p
C4	3.43	3.31	3.40	3.41	3.37	3.40	3.42	0.413
C6	2.02	1.99	2.01	2.02	2.00	2.00	2.10	0.680
C8	1.09	1.09	1.10	1.09	1.08	1.09	1.09	0.942
C10	2.32	2.32	2.35	2.35	2.31	2.31	2.32	0.879
C10:1	0.32	0.32	0.31	0.31	0.32	0.31	0.32	0.622
C12	2.65	2.66	2.66	2.68	2.63	2.64	2.63	0.957
C14	8.79	8.72	8.78	8.75	8.55	8.60	8.66	0.801
C15i	0.19	0.19	0.18	0.19	0.19	0.19	0.20	0.566
C15ai	0.38	0.37	0.38	0.38	0.37	0.37	0.37	0.781
C14:1	0.88	0.88	0.87	0.88	0.86	0.86	0.86	0.702
C15	0.83	0.83	0.82	0.83	0.82	0.81	0.81	0.482
C16i	0.21	0.22	0.21	0.22	0.21	0.21	0.21	0.785
C16	28.20	28.24	28.36	28.48	28.10	28.33	28.38	0.140
C17i	0.39	0.37	0.38	0.38	0.37	0.36	0.37	0.095
C17ai	0.19	0.19	0.19	0.19	0.18	0.19	0.18	0.299
C16:1	1.71	1.66	1.64	1.73	1.65	1.67	1.69	0.080
C17	0.66	0.65	0.65	0.67	0.66	0.65	0.66	0.252
C18	8.46	8.59	8.63	8.46	8.61	8.65	8.56	0.565
C18:1 6-8t	0.25	0.24	0.21	0.22	0.26	0.22	0.22	0.051
C18:1 9t	0.24	0.22	0.20	0.20	0.24	0.20	0.19	0.073
C18:1 10t	0.70	0.66	0.59	0.62	0.70	0.58	0.60	0.082
C18:1 11t	0.81	0.75	0.69	0.71	0.80	0.68	0.68	0.063
C18:1 12t	0.30	0.30	0.26	0.28	0.32	0.26	0.26	0.054
C18: 13t	0.54	0.48	0.46	0.47	0.53	0.46	0.45	0.055
C18:1 9c	25.46	25.75	25.72	25.48	25.74	25.86	25.76	0.816
C18:1 11c	1.02	1.07	1.05	1.04	1.06	1.06	1.05	0.054
C18:1 12c	0.35	0.37	0.37	0.35	0.38	0.36	0.37	0.368
C18: 16t+14c	0.29	0.29	0.29	0.27	0.29	0.26	0.29	0.440
C18:2 9c,12c	3.45	3.47	3.48	3.43	3.49	3.51	3.49	0.311
C20	0.17	0.17	0.16	0.15	0.18	0.16	0.16	0.401
C20:1 9c	0.14	0.13	0.14	0.14	0.14	0.14	0.14	0.889
C18:3 9c,12c,15c	0.42	0.42	0.42	0.41	0.44	0.42	0.42	0.464
C18:2 9c,11t CLA	0.36	0.34	0.35	0.37	0.37	0.37	0.35	0.061
C20:4 AA	0.21	0.21	0.20	0.20	0.21	0.20	0.20	0.500
Total n.i	2.57	2.54	2.51	2.65	2.59	2.60	2.51	0.754
SFA	59.89	59.91	60.25	60.25	59.61	59.97	60.15	0.536
MUFA	32.19	32.34	32.05	31.95	32.46	32.21	32.14	0.693
PUFA	5.26	5.21	5.19	5.15	5.34	5.22	5.20	0.083

302 p, significant differences according to processing ($p<0.05$).

303

Table 2b. Fatty acid composition (g FA/100 g fat) in raw milk and processed by high pressure (250-900); 2nd assay (2A).

	Control	250 MPa	450 MPa	550 MPa	700 MPa	800 MPa	900 MPa	p
C18:1 16t+14c	0.24	0.26	0.25	0.24	0.27	0.24	0.29	0.322
C4	3.53	3.51	3.59	3.62	3.48	3.55	3.55	0.630
C6	2.10	2.27	2.27	2.13	2.08	2.09	2.08	0.509
C8	1.12	1.22	1.20	1.13	1.10	1.11	1.12	0.543
C10	2.37	2.56	2.52	2.39	2.38	2.32	2.35	0.517
C10:1	0.31	0.32	0.32	0.29	0.30	0.29	0.30	0.776
C12	2.66	2.84	2.75	2.65	2.62	2.65	2.61	0.567
C14	**9.82**	10.25	9.91	9.84	9.67	9.83	9.71	0.371
C15i	**0.31**	0.31	0.32	0.31	0.31	0.31	0.30	0.415
C15ai	**0.51**	0.52	0.55	0.51	0.50	0.52	0.50	0.010
C14:1	0.87	0.91	0.91	0.89	0.87	0.88	0.87	0.216
C15	0.96	0.96	0.96	0.95	0.96	0.94	0.95	0.974
C16i	0.32	0.30	0.32	0.31	0.31	0.30	0.30	0.867
C16	**33.40**	33.50	33.02	33.47	33.06	33.48	33.53	0.089
C17i	0.40	0.41	0.42	0.46	0.43	0.41	0.43	0.416
C17ai	**0.59**	0.57	0.58	0.60	0.57	0.59	0.56	0.569
C16:1c	1.53	1.55	1.55	1.57	1.52	1.54	1.50	0.355
C17	**0.37**	0.16	0.14	0.15	0.17	0.17	0.17	0.520
C18	**9.83**	9.46	9.84	9.77	10.10	9.94	9.98	0.433
C18:1 6-8t	0.28	0.28	0.30	0.28	0.27	0.28	0.28	0.601
C18:1 9t	0.26	0.26	0.27	0.26	0.26	0.26	0.27	0.839
C18:1 10t	**0.37**	0.36	0.37	0.35	0.36	0.37	0.38	0.442
C18:1 11t	0.84	0.81	0.83	0.83	0.84	0.85	0.83	0.441
C18:1 12t	0.31	0.31	0.31	0.30	0.32	0.33	0.31	0.434
C18:1 13t	0.63	0.61	0.60	0.61	0.61	0.63	0.63	0.697
C18:1 9c	**19.94**	19.09	19.58	19.94	20.26	19.72	19.59	0.462
C18:1 11c	0.62	0.65	0.63	0.63	0.67	0.62	0.63	0.570
C18:1 12c	0.38	0.36	0.38	0.33	0.37	0.33	0.35	0.467
C18:2 9c,12c	**2.38**	2.29	2.30	2.33	2.33	2.34	2.36	0.520
C20	0.20	0.17	0.19	0.16	0.20	0.18	0.18	0.574
C20:1 9c	0.17	0.16	0.18	0.14	0.17	0.16	0.35	0.422
C18:3	0.48	0.52	0.51	0.47	0.47	0.47	0.46	0.191
C18:2 9c,11t CLA	**0.51**	0.47	0.50	0.48	0.50	0.48	0.32	0.503
C20:4 AA	0.19	0.19	0.19	0.17	0.20	0.18	0.20	0.614
Total n.i	1.22	1.62	1.43	1.44	1.44	1.65	1.75	0.352
SFA	**68.49**	68.99	68.57	68.45	67.96	68.40	68.31	0.550
MUFA	**26.73**	25.92	26.50	26.67	27.10	26.50	26.59	0.570
PUFA	**3.56**	3.47	3.49	3.44	3.50	3.46	3.35	0.783

p, significant differences according to processing ($p<0.05$). Bold concentrations in control milk as significant differences ($p<0,05$) according to seasson.

Table 1. Samples of the present study.

Sample	MPa	Min.	ºC	Volume (ml)
1	0	--	15	500
2	250	5	15	500
3	450	5	15	500
4	550	5	15	500
5	700	5	15	500
6	800	5	15	500
7	900	5	15	500

Table 3. High pressure effects in the phospholipids distribution of the analyzed samples (Assay 1, 1A).

%	Control	250 MPa	450 MPa	550 MPa	700 MPa	800 MPa	900 MPa	p
PE	35,84^{a}	34,95^{a}	30,17^{b}	30,77^{b}	29,52^{b}	20,64^{c}	37,97^{a}	0,000
PI	12,51^{a}	9,95^{b}	9,06^{b}	8,31^{b}	9,14^{b}	13,31^{a}	12,75^{a}	0,000
PS	10,16^{a}	9,11^{a}	7,27^{b}	7,88^{b}	7,41^{b}	9,51^{b}	10,40^{a}	0,003
PC	21,40^{a}	23,19^{ab}	24,52^{b}	24,82^{b}	25,04^{b}	26,92^{b}	19,08^{b}	0,002
SP	20,09^{a}	22,81^{a}	28,98^{b}	28,22^{b}	28,88^{b}	29,63^{b}	19,80^{a}	0,000

p, significant differences according to processing ($p<0.05$).

Table 3b. High pressure effects in the phospholipids distribution of the analyzed samples (Assay 2, 2A).

%	Control	250 MPa	450 MPa	550 MPa	700 MPa	800 MPa	900 MPa	p
PE	**37,82**^{a}	39,62^{a}	41,60^{b}	38,85^{a}	41,87^{b}	41,99^{b}	41,60^{b}	0,001
PI	**4,18**^{a}	1,78^{b}	1,02^{c}	3,95^{a}	1,14^{c}	1,58^{b}	1,54^{b}	0,000
PS	**7,22**^{a}	4,80^{b}	4,30^{b}	7,04^{a}	3,62^{d}	4,72^{b}	4,51^{b}	0,000
PC	**31,21**	32,27	33,28	31,21	33,26	32,57	32,73	0,110
SP	**19,58**	21,52	19,79	18,95	20,11	19,14	19,62	0,173

p, significant differences according to processing ($p<0.05$). Bold concentrations in control milk as significant differences ($p<0,05$) according to seasson.

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3.4. Determinación de la posible alteración de la fracción lipídica de fórmulas infantiles en polvo y derivados lácteos de alto contenido en CLA durante su periodo de conservación.

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Changes in the Lipid Composition of Powdered Infant Formulas during Long-Term Storage

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Changes in the lipid composition of two standard infant formulas induced by 4 years of storage were determined. Lipids were thoroughly analyzed using different gas−liquid and liquid−liquid chromatographic techniques. Oleic acid and linoleic acid, which accounted for almost the total monounsaturated and polyunsaturated fatty acids, respectively, showed slight but significant decreases ($P < 0.05$) during the 4 years of storage (from 41.52 to 39.83% for oleic acid and from 17.35 to 15.99% for linoleic acid). Total trans fatty acid isomers showed low initial level (0.22% of total fatty acids), and such level remained unchanged during the storage period. Nonvolatile oxidation compounds including oxidized, dimeric, and polymeric triglycerides did not significantly increase during the storage period, although a significant loss of tocopherols was found in the surface oil fraction (10−15%). In general, the results obtained indicate that, although small losses of oleic and linolenic acid as well as tocopherols were found, the 4 year storage period did not lead to relevant changes in the lipid fraction of infant formulas.

KEYWORDS: Infant formula; storage; PUFA; trans fatty acids; oxidation compounds

INTRODUCTION

Data accumulated from scientific and clinical studies on long-chain polyunsaturated fatty acids (LCPUFAs) in human milk and as additives to infant formulas suggest that LCPUFAs are essential for energy and growth, organ differentiation and function, and cellular metabolism of preterm and term infants (*1*). Among LCPUFAs, docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are found in human milk. DHA and AA, which are formed from essential fatty acids (EFA), linoleic acid (LA, 18:2n-6), and α-linolenic acid (ALA, 18:3n-3) are rapidly accrued in the central nervous system and retina during the last trimester of pregnancy and the first postnatal year (*2*−*5*).

During the commercial preparation of most infant formulas, the butterfat of whole cow's milk is replaced by a blend of vegetable oils, added to bring the fatty acid composition, especially of polyunsaturated fatty acids, closer to that in human milk (*6*). The formulated raw material mix is blended, pasteurized, homogenized, concentrated, and spray-dried or sterilized, and many resources are used by manufacturers to ensure that products are of good quality and show a long shelf life.

Adequate LCPUFAs contents are achieved mainly by a number of highly unsaturated dietary lipid sources currently available, such as fish oils and oils obtained from fungal and algal organisms. Increases in LCPUFAs contents and parallel mineral fortification as well as technological treatments and long-term storage periods may lead to oxidative modifications with subsequent loss of essential fatty acids, trans fatty acid (TFA) formation, release of volatile compounds responsible for rancidity, and formation of nonvolatile oxidation compounds that may be detrimental to health. Therefore, the oxidation stability of powder formulas and their shelf life clearly depend in great part on the LCPUFAs content, storage temperature, and storage time (*7*).

With respect to TFA, a review of the literature shows little information on their influence in infants (*8*) and on the composition and levels in infant formulas (*9*). On the other hand, changes in lipid oxidation parameters during storage of infant formulas have been reported in a few studies, including monitoring of primary or secondary oxidation products determined by classical indices (*10*−*13*), or stability tests (*14*), as well as monitoring of tocopherol losses (*10*, *15*, *16*). Still, results obtained are often confusing, in part due to the use of methods applicable to only particular stages of the oxidation process.

The aim of the present study was to analyze the effects of long-term storage on infant formulas available on the market and to follow lipid changes, with special focus on the content

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Table 1. Composition of Infant Formulas (Values Were Obtained from the Company and Based on Label Claims)

	baby 1 formula[a]		baby 2 formula[b]	
	100 g powder	100 mL	100 g powder	100 mL
energy (kcal)	522	68	500	70
protein (g)	10.2	1.3	15	2.1
carbohydrate (g)	55.2	7.2	53.7	7.5
fat (g)	29.0	3.8	25.0	3.5

[a] Adapted formula. [b] Follow-on formula. Provided by Hero España, S.A.

Table 2. Ingredients of Infant Formulas (Values Were Obtained from the Company and Based on Label Claims)[a]

ingredient	baby 1 formula	baby 2 formula
skim milk	+	+
demineralized milk whey	+	+
vegetable oils	+	+
lactose	+	+
minerals	+	+
lecithin	+	+
vitamins (A, C, E, B_1, B_2, B_6, B_{12}, D_3, K, niacin, pantothenic acid, folic acid)	+	+
choline	+	+
taurine	+	+
inositol	+	+
carnitine	+	+
amino acids (arginine, histidine, tryptophan)	+	
maltodextrins		+

[a] + indicates the presence of the ingredient.

of PUFAs and TFA, as well as the concentration of oxidation compounds, to determine if such storage could have adverse effects.

MATERIALS AND METHODS

Samples. Two milk-based adapted infant formulas: initiation formula (baby 1) and follow-on formula (baby 2) were provided by a local industry (Hero España, S.A.). Baby 1 formula was designed to meet the nutritional needs of infants from birth, and baby 2 formula was designed for the development and growth of infants up to the age of 4 months. Composition of the formulas is showed in **Table 1**. Four batches of each formula at four storage periods during the shelf life of this product (1−4 years) (32 samples in total) were evaluated in duplicate. First-year samples corresponded to samples received from the company after manufacture and analyzed during the first year of storage. The samples, packed in 900 g sealed metal containers blanketed with inert atmosphere, were stored at ambient temperature (25 ± 3 °C) during 4 years.

Both baby 1 and baby 2 infant formulas were prepared following the same technological process, as follows: milk was skimmed, pasteurized (72 °C/15 s), and concentrated (falling film evaporator at 85, 66, and 58 °C during 5 min). Then pasteurized whey (72 °C/15 s), lipids (a blend of vegetable oils), lactose, and minerals were added, and the formulas were mixed and sterilized (HTST 100 °C/22 s). Finally, the rest of the ingredients (see **Table 2**) were added and spray-dried by atomization (air input at 175−185 °C; air output at 90−94 °C).

Lipid Extraction. For analysis of fatty acid methyl esters (FAMEs) and TFA, lipids were extracted following a procedure described by an International Standard Method for milk powder, ISO-IDF (*17*). Briefly, it consists of the addition of an ammoniacal ethanolic solution to a test portion followed by lipid extraction using diethyl ether and hexane. Then, the upper layer is removed, and the solvent is completely evaporated. The lipid extracts obtained were stored in amber glass vials, exposed to a stream of nitrogen and frozen at −20 °C until analysis. For analyses of oxidized compounds and tocopherols, the fraction of free oil was additionally extracted. The free oil fraction, also known as the nonencapsulated oil fraction, was determined according to the method of Sankarikutty et al. (*18*). Thus, 200 mL of light petroleum ether (60−80 °C) was added to 4 g of powder sample. Stirring was applied at room temperature for 15 min. After filtration through a filter paper, the solvent was evaporated in a rotary evaporator, and the extracted oil was dried to constant weight using a stream of nitrogen.

Preparation of FAME. FAMEs were prepared by base-catalyzed methanolysis of the extracted lipids using 2 N KOH in methanol as described by International Standard ISO-IDF (*19*).

Standards. For GC-FID analysis, anhydrous milk fat with a certified fatty acid composition (reference material BCR-164, EU Commission, Brussels, Belgium, purchased from Fedelco Inc., Madrid, Spain) was used to determine the FAME response factors. For quantitative purposes glyceryl tritridecanoate (Sigma Chemical Co., St. Louis, MO) was also used as internal standard.

Tentative identification of *trans*-C18:2 and *trans*-C18:3 isomers was done by comparing the equivalent chain-length values of FAME obtained with those of reference oils: partially isomerized linseed oil FAME, refined rapeseed oil (BCR 686), partially hydrogenated sunflower seed oil (BCR-688), and a blend of palm oil and partially hydrogenated sunflower seed oil (BCR-687), which had served as test material in the research project SMT4-CT97-2144 of the European Union. Besides this test material, FAME pure isomers (C18:1: *cis*-9; *cis*-13; *trans*-9; *trans*-11; *trans*-13) and polyunsaturated fatty acid mixtures (C18:2 mixture: *trans*-9 *trans*-12 + *cis*-9 *trans*-12 + *trans*-9 *cis*-12 + *cis*-9 *cis*-12; C18:3 mixture: *trans*-9 *trans*-12 *trans*-15 + *trans*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *trans*-15 + *cis*-9 *trans*-12 *trans*-15 + *cis*-9 *cis*-12 *trans*-15 + *cis*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *cis*-15 + *cis*-9 *cis*-12 *cis*-15) supplied by Supelco (Bellefonte, PA) were also used as standards. Octanoic acid methyl ester and elaidic acid methyl ester, supplied by Sigma Chemical Co. (St. Louis, MO), were used as internal standards for TLC analysis.

Silver Argentation Thin Layer Chromatography (Ag^+-TLC). FAMEs were fractionated according to the number and geometry of double bonds by TLC. Briefly, the TLC glass plates (Merck, Darmstadt, Germany) were incubated with 20% aqueous solution of silver nitrate (Panreac, Barcelona, Spain) for 16 h, partially air-dried, and activated at 120 °C for 30 min. A 45 μL solution of FAME (100 mg/mL) was applied in a narrow band, and the plates were developed twice in a saturated chamber containing hexane and diethyl ether (9:1, v/v), leaving 15 cm of migration. At the end of chromatographic runs, the plates were air-dried and sprayed with a 0.20% ethanol solution of 2′,7′-diclorofluorescein (Merck), and the bands were visualized under UV light. The bands were scrapped off and the compounds eluted with diethyl ether and then analyzed by GC as described below.

GC-FID Analysis. FAMEs were analyzed on a Perkin-Elmer chromatograph (Autosystem model, Beaconsfield, U.K.) with a FID detector. Fatty acids were separated using CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, The Netherlands). The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C/min to 170 °C, held there for 55 min, and then temperature-programmed at 10 °C/min to 230 °C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 psi and at a split ratio of 1:20. The injector temperature was set at 250 °C, and the detector temperature was set at 270 °C. Injection volume was 0.5 μL.

Individual trans isomers were analyzed on the same system and column but under other chromatographic conditions: the initial temperature of 100 °C was maintained for 3 min, then raised to 160 °C at a rate of 7 °C/min and held for 62 min, then raised to 220 °C at a rate of 2 °C/min and held for 20 min until the end of the analysis. The split ratio was 1:50, and hydrogen was the carrier gas with a head pressure of 15 psig. The injector and detector temperatures were 250 °C.

Silver Ion HPLC (Ag^+-HPLC). Ag^+-HPLC separation of conjugated linoleic acid (CLA) methyl esters was carried out using an HPLC (Shimadzu Vp Series, Duisburg, Germany) equipped with a UV detector operating at 233 nm. FAMEs were separated using a ChromSpher 5 Lipid analytical column (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Varian-Chrompack International, Middelburg, The Neth-

Table 3. Fatty Acid Composition (Weight Percent on Fatty Acid Methyl Esters) in Infant Formulas during 4 Years of Storage[a]

	years of storage							
	1		2		3		4	
fatty acid[b]	mean	SEM	mean	SEM	mean	SEM	mean	SEM
C4:0	0.07a	0.007	0.07b	0.004	0.06b	0.004	0.08b	0.007
C6:0	0.19a	0.007	0.22b	0.004	0.22b	0.004	0.23b	0.007
C8:0	1.84a	0.032	2.12b	0.035	2.10b	0.014	2.08b	0.035
C10:0	1.32a	0.014	1.51b	0.021	1.49b	0.007	1.50b	0.025
C12:0	10.21a	0.088	11.33b	0.060	11.41b	0.021	11.09b	0.184
C14:0	4.02a	0.021	4.41b	0.025	4.43b	0.018	4.41b	0.085
C15:0	0.04a	0.004	0.04a	0.004	0.04a	0.004	0.05a	0.004
C16:0	16.78a	0.180	16.79a	0.095	16.61a	0.113	16.97a	0.223
C16:1	0.10a	0.004	0.09a	0.004	0.09a	0.004	0.12b	0.004
C17:0	0.05a	0.004	0.05a	0.004	0.05a	0.004	0.06a	0.004
C18:0	3.56a	0.035	3.63a	0.021	3.66a	0.018	3.63a	0.028
trans C18:1 (*t*4 to *t*12)	0.13a	0.014	0.13a	0.007	0.19b	0.011	0.20b	0.018
C18:1 *cis*9	41.52a	0.159	40.50b	0.092	40.10b	0.177	39.83b	0.216
C18:2 *cis*9, *cis*12	17.35a	0.283	16.66ab	0.219	16.91ab	0.145	15.99b	0.170
C20:0	0.27a	0.004	0.26a	0.004	0.26a	0.004	0.26a	0.004
C20:1 *cis*9	0.13a	0.007	0.10a	0.007	0.12a	0.014	0.12a	0.011
C20:1 *cis*11	0.14a	0.007	0.11a	0.007	0.12a	0.007	0.14a	0.018
C18:3n-3	1.49a	0.025	1.16b	0.018	1.15b	0.032	1.05b	0.039
C18:2 conjugated	0.03a	0.004	0.03a	0.004	0.03a	0.004	0.03a	0.004
C20:4n-6	0.30a	0.004	0.32b	0.004	0.33b	0.004	0.29a	0.004
C20:5n-3	0.12a	0.004	0.13a	0.004	0.13a	0.004	0.13a	0.004
∑ SFA	38.3a	0.329	40.4a	0.311	40.3a	0.318	40.6a	0.311
∑ MUFA	42.0a	0.159	40.9b	0.163	40.6b	0.163	40.4b	0.187
∑ PUFA	19.3a	0.286	18.3ab	0.223	18.6a	0.163	17.5b	0.198
∑ n-6	17.7a	0.290	17.0a	0.276	17.3a	0.283	16.3a	0.290
∑ n-3	1.8a	0.028	1.5b	0.028	1.5b	0.028	1.3b	0.028
ratio LA/ALA	11.6		14.3		14.7		15.3	

[a] Data are expressed as means and standard error of the mean (SEM), $n = 8$. Means within the same row with different letters differ ($P < 0.05$). [b] *c* = *cis*; *t* = *trans*; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; LA = linoelic acid; ALA= α-linolenic acid.

erlands). The mobile phase, daily prepared, was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL/min. The flow was initiated 0.5 h prior to the sample injection, and the injection volume was 10 μL. Pure and mixed CLA FAME isomers from Nu-Chek Prep. Inc. (Elysian, MN) were used as standards.

Quantitation of Oxidation Compounds. Quantitative analysis of total nonvolatile oxidation compounds was carried out by separation of polar compounds by solid-phase extraction (SPE) and subsequent analysis by high-performance size-exclusion chromatography (HPSEC) according to the method of Márquez-Ruiz et al. (*20*).

Separation of polar compounds by SPE. A volume of 2 mL of a hexane solution containing 50 mg of extracted lipids and 1 mg of monostearin (Sigma), used as an internal standard, was separated into two fractions by SPE. A first fraction, comprising the unoxidized triglycerides, was eluted with 15 mL of hexane/diethyl ether (90:10, v/v). The second fraction was eluted with 25 mL of diethyl ether and comprises the total nonvolatile oxidation compounds, the internal standard, hydrolytic alteration compounds, that is, diglycerides (DG) and free fatty acids (FFA), and polar unsaponifiable matter. Thus, the oxidation compounds are separated as compounds with higher polarity than that of the nonoxidized triglyceride molecules. After evaporation of the solvent in a rotary evaporator, the polar fraction was dissolved with 1 mL of diethyl ether. The efficiency of the separation was checked by TLC using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) for development of plates and exposure to iodine vapor to reveal the spots.

Analysis by HPSEC. The fraction of polar compounds was analyzed in an HPSEC chromatograph equipped with a Rheodyne injector with a 10 μL sample loop, a Waters 510 pump (Waters, Milford, MA), and a Waters refractive index detector. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm i.d.) packed with porous, highly cross-linked styrene−divinylbenzene copolymers (film thickness = 10 μm) (Agilent Technologies, Palo Alto, CA) connected in series, with tetrahydrofuran (1 mL/min) as the mobile phase. The peaks resolved by HPSEC correspond to triglyceride dimers (TGD), oxidized triglyceride monomers (oxTGM), DG, monostearin, and finally peaks corresponding to FFA of various chain lengths and the polar unsaponifiable matter.

Determination of Tocopherols. Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 (*21*).

Statistical Analysis. For statistical analysis, one-way analysis of variance (ANOVA) as well as multiple-comparisons procedure for each formula type and storage time was used. We conducted statistical analysis utilizing the SPSS package for Windows version 11 (SPSS, Chicago, IL). The level of statistical significance was set at 5% for all analyses.

RESULTS AND DISCUSSION

According to the information supplied by the manufacturer's (listed in **Tables 1** and **2**) infant formulas baby 1 and baby 2 contained the same blend of vegetable oils and were subjected to identical technological process. In fact, the statistical study applied to them confirmed no significant differences between both types of infant formulas with regard to contents of the lipid compounds analyzed in this study for the same storage year. This suggests that differences in other ingredients between the two infant formulas did not affect lipid composition during storage under the conditions used. As a consequence, all of the lipid analysis results obtained from the samples within the same year of storage were joined for statistical analyses.

Fatty Acid Profile. Mean values and standard deviations for fatty acid composition during 4 years of storage are shown in **Table 3**. The major fatty acid present in the infant formulas in quantitative terms was oleic acid (C18:1*cis*9) with around 40% of total FAME, suggesting that rich oleic acid oils, such as the olive oil or high-oleic sunflower oil, could be part of the mixture of vegetable oils used. Likewise, the oleic acid content reported for human milk is in the range of 24−40% (*2*). The level of saturated fatty acids (SFA) in infant formulas was around 40% (also within the range found in human milk of 35−50%), and approximately half of this level was palmitic acid (C16:0). The

Table 4. Trans Monoene Isomer Composition (Milligrams per 100 g of Oil) in Infant Formulas during 4 Years of Storage[a]

	years of storage							
	1		2		3		4	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
C16:1 *trans*	6.91a	0.424	6.20a	0.499	6.56a	0.283	7.36a	0.187
C18:1 *trans*								
trans 4–5	3.88a	0.728	3.39a	0.594	3.19a	0.357	4.05a	0.813
trans 6–8	12.75a	1.538	14.63a	1.294	16.33a	1.895	16.29a	1.146
trans 9	27.37a	1.676	31.20a	1.075	36.11a	1.605	33.22a	1.821
trans 10	38.03a	1.828	38.95a	2.839	43.20a	1.191	42.80a	2.005
trans 11	46.82a	2.386	53.07a	4.221	52.16a	2.669	54.21a	1.446
trans 12	19.07a	1.782	19.89a	1.128	15.91a	1.092	19.55a	0.969
trans 13–14	21.88a	0.933	22.36a	0.760	22.63a	1.255	25.72a	2.669
trans 15	6.41a	0.506	6.16a	0.481	5.88a	0.707	6.45a	0.640
trans 16	7.43a	0.488	6.99a	0.481	7.49a	0.824	7.21a	0.590
C18:1 total *trans* (%)	0.18a	0.007	0.20a	0.007	0.21a	0.007	0.21a	0.007

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$. Means within the same row with different letters differ ($P < 0.05$).

content in medium-chain fatty acids (MCFA C10:0−C14:0) in the infant formulas studied was much higher than that in human milk, especially due to the high content of C12:0 (11% in infant formulas vs 3−8% in human milk). Linoleic acid (LA, C18:2 *cis*9-*cis*12) was the most abundant fatty acid of the PUFA fraction, with values of about 16% (90% of PUFA) and within the range found in human milk (8−18%). Formulas containing large amounts of corn oil have considerably larger contents of LA (>30%), although such high amounts are not recommended for infant formulas (*22*). The content of ALA (C18:3 *cis*9-*cis*12-*cis*15) accounted for approximately 1%. The LA/ALA ratio of the studied formula ranged from 12 to 15, which falls almost within the ratio in human milk and that established by several nutritional recommendations for infant formulas (between 5 and 15). The amounts of very long chain PUFAs were 0.3% of AA and 0.1% of EPA (C20:5) (all in the range found in human milk), although DHA (C22:6) was not detected in the samples studied (present at around 0.3% in human milk).

During the storage period, a gradual decrease in the content of MUFA and PUFA (represented mainly by oleic acid, C18:1*cis*9 and linoleic acid, C18:2 *cis*9-*cis*12, respectively) was observed (**Table 3**). The reduction was especially significant during the first year of storage for oleic and linolenic acid (C18:3 *cis*9-*cis*12-*cis*15), whereas linoleic acid decreased significantly only during the fourth year of storage of the infant formulas studied. Assuming the stability of saturated fatty acids, the normalization of the other fatty acids on the C16:0 amount reveals the absolute losses of 2% of MUFA (mainly oleic acid) and 2% of PUFA (1.5% of LA and 0.5% of ALA). These results are normally attributed to peroxide development during the storage of infant formulas enriched in PUFA (*13*). In this context, data on oxidation compounds and tocopherol levels obtained in this study are discussed below.

As expected, the total CLA content was low (0.3% of total FA), mainly due to the absence of milk fat in these infant formulas, and in agreement with the results of McGuire et al. (*23*). Nevertheless, due to the existing interest in the occurrence of CLA isomers and the lack of available data about its presence in infant formulas, the distribution of these isomers was studied. There are about 20 different CLA isomers in natural milk fat as shown by Ag^+-HPLC separation (*24*). The CLA isomers distribution (in relative proportions of total CLA) accounted for 38% of C18:2 *trans*,*trans* isomers (the major isomers were C18:2 *trans*10,*trans*12 and C18:2 *trans*9,*trans*11 CLA.), 16% of C18:2 *cis*,*cis* isomers, and 46% of C18:2 *cis*,*trans* plus *trans*,*cis* isomers. The most biologically important isomers described are C18:2 *cis*9,*trans*11 (rumenic acid) and C18:2 *trans*10,*cis*12, which accounted for 22 and 8.5% respectively, in the infant formulas analyzed. This isomer distribution did not match with milk fat, where rumenic acid is the most abundant isomer (around 80%) but was in the same range as that reported by Jung and Jung (*25*) for soybean oil determined by the same method.

On the other hand, Juaneda et al. (*26*) reported that the CLA level was positively influenced by deodorization temperature of sunflower oil (total CLA increased 0.2% at 180 °C and 1.3% at 220 °C). These authors also reported that the main CLA isomers found in fresh or heated oils were the C18:2 *trans*,*trans*, mainly 9, 11, and 10, 12, isomers. In this work, it is remarkable that neither the CLA content nor the CLA isomers distribution showed significant differences during the storage period of the studied infant formulas.

Trans Fatty Acid Isomers Content. Determination of total TFA content as well as the amount of individual TFA isomers in infant formulas during the storage period is important due to their possible adverse effects on the health of newborns and infants. The preliminary isolation of trans isomers by Ag^+-TLC is essential due to the complexity of their analysis by direct GC because of the coelution of *trans*-octadecanoic isomers (*t*-C18:1) with oleic acid. Also, the C16:1 *trans* isomer coelutes with C17:0 in milk fat (*27*). Normally, the vegetable oils used in infant formulas are not hydrogenated, and therefore the presence of TFAs is mainly a consequence of the deodorization conditions of the refining process performed at temperatures >200 °C (*28*).

TFAs values detected in the infant formulas studied during 4 years of storage are presented in **Table 4**. Among TFAs, *t*-C18:1 was the main isomer present, whereas *trans*-octadecadienoic (*t*-C18:2) and *trans*-octadecatrienoic acids (*t*-C18:3) were not detected. Isomers of *trans*-C18:1 were well identified and may be used as quality markers for vegetable oils incorporated in infant formulas. The three major *t*-C18:1 isomers, C18:1*t*-11 (vaccenic acid), C18:1*t*-10, and C18:1*t*-9 (elaidic acid), accounted for more than half of the total TFAs, and storage time of up to 4 years did not result in any significant increase. Overall, the total *trans*-C18:1 content was in the range of 0.20% of total fatty acids in infant formulas, 10-fold lower as compared to values reported in human milk (*29*, *30*), and did not change significantly during storage.

Formation of Oxidation Compounds and Loss of Tocopherols. In this study, two complementary approaches have been used to monitor oxidative changes during long-term storage,

Table 5. Oxidized Triacylglycerol Monomers (oxTGM), Triglycerol Dimers (TGD), Diacylglycerols (DG), and Free Fatty Acids (FFA) (Weight Percent on Extracted Lipids) in Total and Free Oil Fractions of Infant Formulas during 4 Years of Storage[a]

		years of storage							
		1		2		3		4	
		mean	SEM	mean	SEM	mean	SEM	mean	SEM
oxTGM	total oil	1.3	0.27	1.1	0.06	1.1	0.06	1.2	0.23
	free oil fraction	1.5	0.08	1.2	0.21	1.1	0.05	1.5	0.23
TGD	total oil	0.5	0.02	0.5	0.03	0.5	0.03	0.5	0.06
	free oil fraction	0.4	0.05	0.5	0.12	0.3	0.11	0.5	0.06
DG	total oil	2.1	0.17	2.0	0.07	2.0	0.15	2.1	0.09
	free oil fraction	2.2	0.26	2.2	0.41	2.2	0.10	2.4	0.09
FFA	total oil	0.6	0.04	0.7	0.02	0.7	0.10	0.6	0.02
	free oil fraction	0.5	0.04	0.6	0.02	0.5	0.03	0.5	0.02

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$.

Table 6. Tocopherols (Toc) (Milligrams per Kilogram of Extracted Lipids) in Total and Free Oil Fractions of Infant Formulas during 4 Years of Storage[a]

		years of storage							
		1		2		3		4	
		mean	SEM	mean	SEM	mean	SEM	mean	SEM
total Toc	total oil	293a	15.6	331a	18.3	317a	14.8	382a	10.3
	free oil fraction	246b	14.3	281b	12.2	282b	7.3	332b	17.6
α-Toc	total oil	151a	9.8	173a	11.3	156a	11.1	188a	7.4
	free oil fraction	109b	14.6	138b	8.7	135a	6.2	160a	11.9
β-Toc	total oil	9a	1.7	11a	2.3	8a	1.4	10a	1.2
	free oil fraction	11a	2.1	13a	1.7	11a	1.9	12a	1.2
γ-Toc	total oil	102a	5.5	97a	15.5	116a	5.8	142a	4.1
	free oil fraction	83b	6.6	96a	4.0	94a	9.6	123b	4.7
δ-Toc	total oil	30a	1.6	51a	13.1	37a	2.1	40a	1.5
	free oil fraction	42a	7.4	34a	2.8	42a	8.4	37a	1.8

[a] Data are expressed as mean and standard error of the mean (SEM), $n = 8$. Values in each column for total oils and free oil fractions in samples of the same time period with different letters are significantly different ($P < 0.05$).

that is, quantitation of nonvolatile oxidation compounds, which include primary and secondary products, and determination of tocopherols, the major antioxidants present in these samples. Additionally, not only were the total oils extracted analyzed but also the minor lipid fraction normally called free or unencapsulated fraction. Isolation of this lipid fraction is achieved by simply washing with hexane (see Materials and Methods). Even though it constituted only 3−7% of total oil in these samples, its analysis is of great relevance because the free oil fraction may be more susceptible to oxidation than the oil fraction embedded in the matrix (encapsulated oil), and rancidity of the free oil fraction is easily perceived by consumers (*31*, *32*).

Table 5 shows data of oxidized triglyceride monomers (oxTGM), triglyceride dimers (TGD), diglycerides (DG), and free fatty acids (FFA) in samples stored for up to 4 years. The group of oxTGM includes the primary oxidation compounds formed (hydroperoxides) and secondary oxidation compounds (alcohols, ketones, epoxides, etc.) in monomeric TG structures, whereas TGD formation marks the start of the advanced oxidation stage (*20*, *33*, *34*). No significant differences were found in oxidation compounds (oxTGM and TGD) or in hydrolysis products (DG and FFA), either in total oils or in free oil fractions, during the storage period. The values reported are within those normally found in refined vegetable oils.

Table 6 shows data of total tocopherols and specific values for α, β, γ, and δ isomers. Infant formulas contain tocopherols derived from the vegetable oils used and from their specific addition during their manufacture. In this study, α-tocopherol and γ-tocopherol constituted the major fraction of the total tocopherol content. European legislation requires a minimum content of 0.5 α-tocopherol equivalents per 100 kcal in infant formulas (*35*). In this study, no significant differences due to storage time were found, and the total vitamin E activity exceeded by far the minimum content established by European law. However, values of total tocopherols were significantly lower in free oil fractions than in total oils independent of the storage time. Accordingly, the data obtained indicate that losses of tocopherols in this fraction were not due to storage conditions but to the manufacturing processes involving thermal conditions (e.g., sterilization and atomization processes) because differences between total and free oil were of the same order independent of the storage time. Although the free oil fraction was still well protected by remaining antioxidants in all samples, this finding clearly suggests that free oil is more susceptible to oxidation than encapsulated oil. These results are first reported here because tocopherols have been only analyzed in the total oil extracted from powdered infant formulas so far (*10*, *15*, *16*).

In conclusion, significant although very small decreases of MUFA (mainly oleic acid) and PUFAs (AA and ALA) were found during the first year of storage of the infant formulas studied. Parallel small losses of other PUFAs, such as LA, were significant only after 3 years of storage. However, formation of oxidation compounds or trans fatty acids was not detected; losses in total tocopherol levels were not significant, which occurs prior to significant oxidation. Therefore, we can conclude that long-term storage did not lead to greatly appreciable lipid changes in the infant formulas studied.

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Hot Topic: Fatty Acid and Conjugated Linoleic Acid (CLA) Isomer Composition of Commercial CLA-Fortified Dairy Products: Evaluation After Processing and Storage

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ABSTRACT

Conjugated linoleic acid (CLA) exerts a strong positive influence on human health but intake of these fatty acids is typically too low, and increased consumption of CLA is recommended. A good way to raise the CLA content in the diet without a radical change in eating habits seems to be the enrichment of commonly consumed food products with CLA supplements. This study analyzed the total fatty acid content and the CLA isomer composition of 6 commercially available CLA-fortified dairy products during processing and 10 wk of refrigerated storage. Research was carried out by combining gas chromatography and silver-ion HPLC. The tested samples were a CLA oil supplement, and several skim milk dairy products fortified with the supplement (milk, milk powder, fermented milk, yogurt, fresh cheese, and milk-juice blend). The CLA oil supplement was added such that the consumer received 2.4 g/d of CLA by consuming 2 servings. The predominant isomers present, C18:2 *cis*-9, *trans*-11 CLA and C18:2 *cis*-10, *trans*-12 CLA, were in at a similar ratio, which ranged from 0.97 to 1.05. These major isomers were not significantly affected by processing but a decrease in total CLA in fresh cheese samples was detected after 10 wk of refrigerated storage. Refrigerated storage and thermal treatment resulted in significant decreases or disappearance of some of the minor CLA isomers and a significant increase of *trans, trans* isomers from both *cis, trans, trans, cis,* and *cis, cis* isomers especially in CLA-fortified milk powder but also in fermented milk, yogurt, and milk-juice blend.
Key words: conjugated linoleic acid, milk product, fatty acid composition

INTRODUCTION

The generic name "conjugated linoleic acid" (**CLA**) is a collective term embracing all octadecadienoic acids (C18:2) with a conjugated double bond system in the 7–9, 8–10, 9–11, 10–12, 11–13, and 12–14 positions and a *cis*, *cis*, *cis*, *trans*, *trans*, *cis*, and *trans*, *trans* geometrical configuration. Recently, evidence has suggested that individual CLA isomers might act differently in biological systems and contribute in different ways in their beneficial or potential side effects (Belury, 2002a; Khanal, 2004; Pariza, 2004; Parodi, 2004; Terpstra, 2004). Data from animal models have been used to suggest that the CLA isomer C18:2 *cis*-9, *trans*-11, also known as rumenic acid (**RA**), is responsible for the anticarcinogenic properties of CLA, as well as growth-promoting and antiatherogenic effects (Ip et al., 1994, 1996, 2002; Belury, 2002b; Masso-Welch, et al., 2002, 2004), whereas the C18:2 *cis*-10, *trans*-12 isomer is responsible for the observed weight loss and muscle-mass enhancement effects (Gaullier et al., 2004; Malpuech-Brugere et al., 2004).

The advantageous nutritional properties and benefits associated with CLA have important implications for food industries whose challenge is the production of functional foods with high health-promoting capacities.

Most full-fat dairy products contain CLA in quantities varying from 6 to 16 mg/g of total fat content, with lesser amounts in meat (Parodi, 1977), 85 to 95% of which is present as the C18:2 *cis*-9, *trans*-11 isomer. Therefore, estimates of CLA daily intake from food sources range from 150 to 212 mg/d (McGuire et al., 1997) or from 300 mg to 1.5 g (Fritsche et al., 1999) although actual intake appears to be dependent on gender and intake of food from animal or vegetable origins. Ip et al. (1994) estimated that a 70-kg human should consume 3.0 g of CLA/d to achieve maximum health benefits. Similarly, CLA supplementation in overweight subjects after weight loss seems to aid the regain of fat-free mass at experimental doses of 1.8 and 3.6 g/d (Kamphuis et al., 2003). Nevertheless, the extrapolation of CLA effects observed in animals to the human situation should be made with caution.

There are different approaches to increasing the human dietary intake of CLA isomers from food. One is to modify the feeding diets of ruminants with supplements

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rich in polyunsaturated fatty acids (**PUFA**) that provide lipid substrates for the production of *cis*-9, *trans*-11 C18:2 or *trans*-11 C18:1 (*trans*-vaccenic acid; Stanton et al., 2003; Khanal and Olson, 2004; Luna et al., 2005b). This strategy has proved to be effective but the concentration of CLA in the milk that is richest in CLA is low compared with other commercial sources of CLA, such as CLA capsules or CLA-fortified dairy products, that provide an additional oral source of CLA to supplement the human diet and complement the CLA amount contained in foods.

The interest in CLA as a nutritional supplement is high and different products are now offered commercially (Sæbø, 2003). Various methods are available to produce synthetic CLA but alkaline isomerization of linoleic acid is the most common (Villeneuve et al., 2005). These commercial supplements contain 50 to 80% CLA and correspond to a complex mixture of isomers, with the C18:2 *cis*-9, *trans*-11 and C18:2 *cis*-10, *trans*-12 isomers being the most abundant, accounting for approximately 90% at a 1:1 level, with the remaining isomers consisting of all *cis*- and all *trans*-isomers of 9,11-, 10,12-, and 11,13-C18:2 (Ma et al., 1999).

Studies of CLA-fortified products, including their behavior during production and storage, would support the development of consumer-acceptable strategies and processing systems to produce CLA-enriched products and enhanced dairy foods of proven quality.

Although Campbell et al. (2003) prepared and compared 3 samples of fluid milk containing 2% total fat (2% milk fat; 1% CLA oil:1% milk fat; and 2% CLA oil) on the sensory, chemical, and physical characteristics, the total fatty acid compositions, including CLA and CLA isomer distribution, have not been examined in commercially available CLA-fortified dairy products.

In the present study, a CLA oil supplement and commercial CLA-fortified dairy products were analyzed for CLA isomers and total fatty acid composition using a gas chromatographic method combined with a silver ion (Ag^+)-HPLC method. To our knowledge, this study demonstrated for the first time significant differences among commercially available CLA-fortified dairy products and their evaluation during processing and storage.

The results of the effect of processing conditions, storage, and aging on the CLA content of various types of dairy products are unclear. With regard to cheeses, reports and reviews present results for individual varieties, often in the belief that CLA levels may vary due to different processing conditions. Herzallah et al. (2005) reported CLA decreases of 21 and 53% in cheeses heated in a microwave oven for 5 and 10 min, respectively. Nevertheless, these effects are likely to be small, and variations in CLA levels are similar to the levels in the starting milk (Shantha et al., 1995; Dhiman et al., 1999; Gnädig and Sébédio, 2002; Luna et al., 2005c). However, other studies detected new CLA isomers in ripened cheeses (Werner et al., 1992; Lavillonière et al., 1998; Sehat et al., 1998) and it was hypothesized that biohydrogenation of linolenic acid in cheese could lead to the formation of CLA isomers as intermediates.

In this article, industrial-scale production of milk products enriched with a commercial CLA oil supplement was conducted. Fatty acid composition and CLA isomer profiles were determined to investigate their development during processing and 10 wk of storage. Our aim was to determine whether there were any compositional differences in these products containing high concentrations of healthy fatty acids.

MATERIALS AND METHODS

Samples and Standards

Five commercially available CLA-fortified dairy products in Spain (milk, fermented milk, yogurt, fresh cheese, milk-juice blend) and a CLA-fortified milk powder (noncommercial) were shipped from the manufacturer (Capsa Inc., Oviedo, Spain) to the laboratory in isothermal containers at 4°C. Samples were maintained at this temperature until fat extraction was carried out. Each product consisted of a different concentration of a CLA oil added as a supplement such that the consumer received 2.4 g/d of CLA when consuming the recommended 2 servings (Table 1). Skim milk with CLA oil added was dual-homogenized (20,000 ± 1,000 kPa) and treated by an indirect UHT process at 142°C for 6 s. Milk powder was obtained after atomization of the CLA-enriched milk sample. Fermented milk and yogurt with CLA oil added were dual-homogenized and HTST-pasteurized at 95°C, and traditional yogurt cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) were added. Fresh cheese was made from skim milk with CLA oil, salt, and rennet; no starter was added. Milk-juice blend (20% fruit from concentrate) was made from skim milk with CLA oil added, pasteurized at 100°C for 30 s, and single-homogenized. A sample of the CLA oil (Tonalin-80, Cognis, Düsseldorf, Germany) used to fortify the milk products was also donated by the manufacturer and analyzed. A total of 9 samples from each product (3 samples at 3 times: 1, 5 and 10 wk of storage at 4°C) were analyzed except for the CLA oil supplement and milk powder, for which storage studies were not carried out.

Pure and mixed CLA isomer methyl esters (C18:2 *cis*-9, *trans*-11 CLA and C18:2 *cis*-10, *trans*-12 CLA) were purchased from Nu-Chek Prep (Elysian, MN). For quantitative determinations of total fatty acid methyl esters (**FAME**), an anhydrous milk fat (reference mate-

Table 1. Product information of the different fortified dairy products studied

Component	Product					
	Milk	Milk powder	Fermented milk	Yogurt	Cheese	Milk-juice blend
pH	6.7	6.7	4.0	4.6	6.5	3.7
Fat, %	1.0	10	2	1.6	1.5	0.8
Protein, %	3.2	32	2.6	5.0	13.4	0.3
Tonalin,[1] %	0.6	6	1.5	1.2	1.2	0.6
Starter culture	No	No	Yes	Yes	No	No
Serving size, g	250	25	100	120	125	250
CLA[2] per serving, g	1.2	1.2	1.2	1.2	1.2	1.2

[1]Tonalin-80, Capsa Inc., Oviedo, Spain; a CLA-supplemented oil.
[2]Conjugated linoleic acid.

rial BCR-164; EU Commission; Brussels, Belgium, purchased from Fedelco Inc., Madrid, Spain) was used. An internal standard (12.4 mg/mL of C13:0 as triacylglyceride; Sigma, St. Louis, MO) was also used.

Lipid Extraction and Fatty Acid Derivatization

Milk fat extraction was carried out according to standard methods (ISO-IDF, 2001). The fat residue extracted was stored frozen at –20°C until analysis. Fatty acid methyl esters were prepared by base-catalyzed methanolysis of the glycerides (2 *N* KOH in methanol) according to standard methods (ISO-IDF, 2002).

Gas Chromatography–Flame-Ionization Detection Analyses

Fatty acid methyl esters were analyzed on a Perkin-Elmer chromatograph (model Autosystem, Beaconsfield, UK) with a flame-ionization detector (**FID**). Fatty acid methyl esters were separated using a fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, CP-Sil 88, Chrompack, Middelburg, the Netherlands). The column was held at 100°C for 1 min after injection, then the temperature was increased at 7°C/min to 170°C, held for 55 min, then increased at 10°C/min to 230°C, and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 214 kPa (30 Psig) and a split ratio of 1:20. The injection volume was 0.5 μL. The CLA isomers were determined and identified by GC-FID by comparing the supplement CLA oil and CLA standards, in accordance with our laboratory's previous studies (Luna et al., 2005a).

Silver Ion-HPLC

Silver ion (Ag^+)-HPLC separation of CLA methyl esters was carried out using an HPLC (Shimadzu Vp Series, Duisburg, Germany) equipped with a UV detector operated at 233 nm. Fatty acid methyl esters were separated using an analytical column (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; ChromSpher 5 Lipid column, Varian-Chrompack Int., Middelburg, the Netherlands). The mobile phase was 0.1% acetonitrile in hexane, operated isocratically at a flow rate of 1.0 mL/min. The flow was initiated 0.5 h before the sample injection and the injection volume was 10 μL. Pure and mixed CLA FAME isomers from Nu-Chek Prep were used as standards.

Statistical Analysis

Data were analyzed using the ANOVA procedure of the SPSS package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL). Multiple range tests were applied to determine significance between different treatments.

RESULTS AND DISCUSSION

FAME Composition of CLA-Enriched Dairy Products by GC-FID

Table 2 shows FAME percentages in the CLA oil sample (Tonalin) and in 6 CLA-enriched products after 1, 5, and 10 wk of refrigerated storage. As expected, commercial CLA oil supplements typically comprised 2 major isomers: C18:2 *cis*-9, *trans*-11 CLA and C18:2 *cis*-10, *trans*-12 CLA. Minor CLA isomers detected by GC-FID (Figure 1) included *cis*-11, *trans*-13; *cis*-9, *cis*-11; and *cis*-10, *cis*-12. The *trans*-8, *cis*-10 isomer is included in the *cis*-9, *trans*-11 peak. Other minor CLA isomers in the chromatographic area studied included an overlapping of at least 3 *trans/trans* isomers that eluted as a single peak (*trans*-8, *trans*-10; *trans*-9, *trans*-11; and *trans*-10, *trans*-12).

The main differences in the FAME profiles between the analyzed products were related to their CLA content. The CLA oil supplement contained around 80% of total CLA whereas the supplemented samples contained in the range of 50 to 75% of total CLA, due to

Table 2. Fatty acid composition of conjugated linoleic acid (CLA) oil supplement and CLA-fortified products, after 1, 5, and 10 wk of refrigerated storage

Sample[1] (wk of storage)	Fatty acids,[2] % of total fatty acids									
	C16:0	C18:0	C18:1 c9	C18:2 c9, c12	C18:2 c9, t11	C18:2 t10, c12	SFA	MUFA	PUFA	CLA
CLA oil supplement	0.20 ± 0.02	2.54 ± 0.12	13.37 ± 0.57	0.43 ± 0.06	39.65 ± 0.76	38.12 ± 0.76	2.82 ± 0.13	14.12 ± 0.79	82.37 ± 1.07	81.27 ± 1.04
Milk powder	7.37 ± 0.26	4.14 ± 0.15	13.21 ± 0.45	0.47 ± 0.07	31.11 ± 0.79	32.03 ± 0.69	18.17 ± 0.54	15.33 ± 0.69	66.12 ± 0.94	64.71 ± 0.84
Milk										
1	11.68 ± 0.61	5.46 ± 0.21	17.52 ± 0.67	1.31 ± 0.17	24.61 ± 1.83	23.55 ± 1.14	26.61 ± 1.85	20.37 ± 1.31	52.41 ± 3.32	49.91 ± 0.77
5	12.14 ± 0.76	5.24 ± 0.35	15.80 ± 0.94	1.32 ± 0.25	24.45 ± 0.73	24.05 ± 0.67	26.51 ± 1.15	18.58 ± 1.47	53.70 ± 1.53	50.70 ± 0.52
10	11.87 ± 0.93	5.15 ± 0.14	16.80 ± 0.49	1.39 ± 0.18	24.01 ± 0.56	23.50 ± 0.47	26.09 ± 0.73	19.88 ± 1.37	52.71 ± 1.41	49.69 ± 0.85
Fermented milk										
1	1.62 ± 0.17	2.54 ± 0.20	14.55 ± 0.48	0.35 ± 0.02	38.03 ± 0.74	37.75 ± 0.39	4.98 ± 0.17	15.51 ± 0.59	79.01 ± 0.66	77.34 ± 0.99
5	1.63 ± 0.12	2.33 ± 0.22	14.62 ± 0.31	0.36 ± 0.04	37.90 ± 0.67	37.42 ± 0.57	4.76 ± 0.14	15.65 ± 0.44	78.94 ± 0.58	77.19 ± 0.89
10	1.75 ± 0.19	2.44 ± 0.18	14.74 ± 0.53	0.37 ± 0.03	37.21 ± 0.86	37.00 ± 0.35	5.08 ± 0.21	15.86 ± 0.58	78.19 ± 0.82	76.52 ± 0.76
Yogurt										
1	5.96 ± 0.83	3.79 ± 0.34	16.18 ± 0.52	0.76 ± 0.15	32.10 ± 1.00	31.11 ± 1.61	14.24 ± 0.93	17.80 ± 0.51	67.34 ± 2.07	65.22 ± 2.07
5	5.27 ± 0.56	3.45 ± 0.50	15.68 ± 1.69	0.68 ± 0.13	32.68 ± 2.10	32.28 ± 0.71	13.60 ± 0.68	17.46 ± 1.73	68.60 ± 1.17	66.58 ± 1.30
10	6.08 ± 0.50	3.77 ± 0.66	15.36 ± 1.86	0.77 ± 0.21	32.12 ± 3.20	31.28 ± 0.81	14.66 ± 0.44	17.17 ± 1.15	67.42 ± 1.27	65.28 ± 1.54
Cheese										
1	1.82 ± 0.12[a]	2.82 ± 0.16[a]	16.49 ± 1.01	0.63 ± 0.16	36.81 ± 0.95	34.79 ± 1.43	5.48 ± 0.38[a]	17.58 ± 1.16	76.57 ± 0.82[a]	75.34 ± 0.92[a]
5	3.78 ± 0.19[b]	3.78 ± 0.29[b]	15.06 ± 1.31	0.62 ± 0.12	36.73 ± 1.30	34.14 ± 1.22	7.48 ± 0.47[b]	16.29 ± 1.19	74.82 ± 0.89[a]	74.41 ± 1.53[a]
10	3.30 ± 0.12[b]	3.42 ± 0.13[b]	16.47 ± 0.73	0.82 ± 0.21	34.59 ± 1.47	32.86 ± 1.15	8.42 ± 0.54[b]	18.01 ± 1.14	72.32 ± 1.33[b]	70.85 ± 1.26[b]
Milk-juice blend										
1	2.37 ± 0.22	5.02 ± 0.45	13.71 ± 0.81	0.37 ± 0.11	37.50 ± 0.94	36.83 ± 0.96	8.08 ± 0.54	14.46 ± 1.44	77.46 ± 1.21	76.22 ± 1.24
5	2.69 ± 0.52	3.98 ± 0.50	12.25 ± 1.04	0.30 ± 0.15	39.14 ± 1.08	37.69 ± 1.15	7.85 ± 0.26	12.94 ± 1.10	78.83 ± 1.10	78.03 ± 1.37
10	2.42 ± 0.15	4.89 ± 0.53	14.03 ± 0.84	0.40 ± 0.07	36.72 ± 1.29	36.71 ± 0.51	8.36 ± 0.36	14.92 ± 0.63	76.69 ± 1.32	75.61 ± 1.31

[a,b]Means within a product and column with different superscripts differ ($P < 0.05$).

[1]The CLA oil supplement and milk powder were not evaluated over the storage period.

[2]c = *cis*; t = *trans*; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

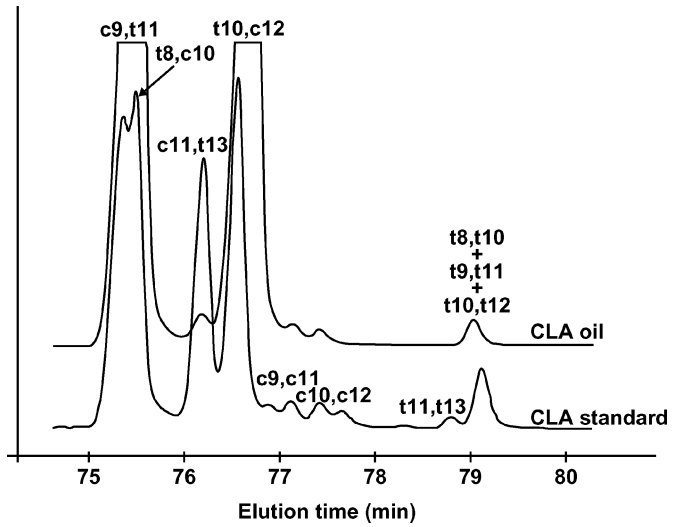

Figure 1. Fatty acid methyl ester profile by gas chromatography with flame-ionization detection of conjugated linoleic acid (CLA) oil used as a supplement to fortify the studied dairy products and profile of the standard CLA methyl ester mixture (Nu-Chek Prep Inc., Elysian, MN). c = *cis*, t = *trans*.

the presence of individual non-CLA fatty acids. Thus, the lower occurrence of CLA in some of the samples, such as milk and yogurt (50 and 65%, respectively) was correlated with the higher presence of milk fat fatty acids. The percentage of saturated fatty acids (**SFA**) in milk and yogurt (26 and 14%, respectively), and of short- and medium-chain fatty acids (2.5 and 6.9% in milk; 1.2 and 3.2% in yogurt, respectively) was high, which is related to the presence of milk fat in the products. The occurrence of milk fat fatty acids in some of the studied products (especially in milk) was higher than would be expected in a skim milk.

The high ratio of SFA to PUFA in dairy fats is undesirable from a nutritional perspective due to the link between saturated fats and increased levels of serum cholesterol and heart disease. Nevertheless, in all of the products studied, C18:2 *cis*-9, *trans*-11 and C18:2 *cis*-10, *trans*-12 CLA were the predominant fatty acids present. All samples had a similar ratio of C18:2 *cis*-9, *trans*-11 to *trans*-10, *cis*-12 CLA, which ranged from 0.97 to 1.05. Moreover, in these studied samples, the ratio of CLA to PUFA was greater than 0.95 in all cases. In addition to CLA, oleic acid (C18:1 *cis*-9) was the most abundant fatty acid (around 15%) and linoleic acid (C18:2 *cis*-9, *cis*-12) was also present, giving all of the studied dairy products a balanced SFA to PUFA ratio.

As mentioned earlier, other minor CLA isomers did not occur as pure chromatographic peaks but were overlapped as indicated by a single peak (*trans*-8, *trans*-10; *trans*-9, *trans*-11; and *trans*-10, *trans*-12; Figure 1). These compounds were detected in all products at levels ranging from 1.6 to 3.5%.

Effects of Processing and Refrigerated Storage of Fatty Acid Composition by GC

Due to the existence of variable amounts of milk fat present in the CLA-fortified samples, comparisons of the fatty acid content between CLA oil and CLA-enriched samples immediately after preparation and treatment could not be determined by GC analysis. Instead, it was studied by Ag^+-HPLC of the total CLA fraction and individual isomers as discussed below.

Results obtained throughout the refrigerated storage of CLA-fortified dairy products for 1, 5, and 10 wk showed that significant differences were found only in the fresh cheese sample. Both C18:2 *cis*-9, *trans*-11 CLA and C18:2 *cis*-10, *trans*-12 CLA isomers decreased but not significantly after 5 wk of storage; subsequently, a significant loss of the total CLA and total PUFA fraction occurred within the same period of storage (Table 2). Campbell et al. (2003) found a significant loss of C18:2 *cis*-9, *trans*-11 CLA after HTST pasteurization of 2% CLA-fortified skim milk. The same authors reported a significant decrease of C18:2 *cis*-9, *trans*-11 after 3 wk of refrigerated storage compared with the levels of this isomer at 1 and 2 wk of storage. These reductions of C18:2 *cis*-9, *trans*-11 were attributed to the heat processing and to excessive microbial growth during the storage of the milk samples.

In the fermented milk studied in this work (a CLA-enriched sample with yogurt culture), declines of C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 CLA were also detected after wk 5 of storage, but were not statistically significant. Nevertheless, no similar decreases were found in yogurt. No change was observed in milk or milk-juice blend samples during storage. Xu et al. (2005) demonstrated that the combination of most probiotic bacteria with the yogurt cultures produced slightly higher contents of C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 CLA, but it did not occur in yogurt culture alone (i.e., without probiotic bacteria) after 14 d of storage.

CLA Isomer Composition of CLA-Enriched Products by Ag^+-HPLC

The use of Ag^+-HPLC is currently the most effective way of separating and quantifying CLA isomers. Conjugated linoleic acid FAME are selectively detected by their characteristic UV absorbance at 233 nm; nonconjugated FAME respond poorly at this wavelength. There are about 20 different CLA isomers in natural milk fat based on Ag^+-HPLC separation (Sehat et al., 1998).

Table 3. Conjugated linoleic acid (CLA) isomer composition of CLA oil supplement and CLA-enriched products after 1, 5, and 10 wk of storage

Sample (wk of storage)	CLA isomer composition,[1] % of total CLA							
	t11, t13	t10, t12	c11, t13	t10, c12	c9, t11	t8, c10	c9, c11	c10, c12
CLA oil supplement	0.56 ± 0.06[x]	0.49 ± 0.05[x]	1.65 ± 0.23[x]	45.74 ± 1.84	48.57 ± 0.61	1.76 ± 0.32[x]	0.54 ± 0.16	0.55 ± 0.08
Milk powder	0.99 ± 0.09[y]	0.89 ± 0.07[y]	0.24 ± 0.04[y]	47.81 ± 1.14	48.48 ± 0.45	0.31 ± 0.18[y]	0.59 ± 0.08	0.57 ± 0.07
Milk								
1	0.65 ± 0.12	0.63 ± 0.12	1.49 ± 0.28	44.96 ± 1.85	49.46 ± 1.88	1.65 ± 0.28	0.51 ± 0.15	0.52 ± 0.10
5	1.10 ± 0.45	1.04 ± 0.49	1.64 ± 0.15	46.12 ± 1.57	47.28 ± 1.31	1.49 ± 0.52	0.43 ± 0.09	0.58 ± 0.05
10	1.08 ± 0.45	1.03 ± 0.46	1.37 ± 0.11	47.58 ± 1.70	45.91 ± 1.67	1.82 ± 0.13	0.23 ± 0.08	0.53 ± 0.04
Fermented milk								
1	0.43 ± 0.06	0.38 ± 0.05	0.11 ± 0.12[y]	49.45 ± 1.82	48.35 ± 1.90	0.25 ± 0.03[y]	0.50 ± 0.06	0.51 ± 0.05
5	0.43 ± 0.06	0.37 ± 0.04	0.29 ± 0.10	47.82 ± 1.42	49.82 ± 1.82	0.24 ± 0.04	0.50 ± 0.05	0.51 ± 0.04
10	0.45 ± 0.04	0.41 ± 0.03	0.34 ± 0.20	47.78 ± 1.02	49.11 ± 1.75	0.27 ± 0.05	0.45 ± 0.08	0.45 ± 0.07
Yogurt								
1	0.58 ± 0.20	0.54 ± 0.11	ND	46.18 ± 1.07	49.30 ± 0.64	1.11 ± 0.15	0.48 ± 0.25	0.61 ± 0.13
5	0.44 ± 0.17	0.39 ± 0.17	ND	48.51 ± 1.47	48.72 ± 0.46	1.23 ± 0.11	0.47 ± 0.18	0.51 ± 0.07
10	0.55 ± 0.14	0.48 ± 0.13	ND	46.77 ± 1.87	49.31 ± 0.69	1.05 ± 0.15	0.39 ± 0.10	0.49 ± 0.14
Cheese								
1	0.53 ± 0.06	0.46 ± 0.10	2.38 ± 0.27[a]	44.01 ± 0.70	49.09 ± 0.49	2.33 ± 0.30	0.56 ± 0.15	0.61 ± 0.09
5	0.69 ± 0.05	0.67 ± 0.05	1.38 ± 0.21[b]	47.03 ± 0.51	48.49 ± 0.66	1.75 ± 0.26	ND	ND
10	0.70 ± 0.08	0.62 ± 0.14	1.42 ± 0.14[b]	46.88 ± 0.89	48.48 ± 0.53	1.85 ± 0.15	ND	ND
Milk-juice blend								
1	0.52 ± 0.04	0.47 ± 0.04	ND	47.40 ± 1.27	49.80 ± 2.04	0.55 ± 0.15[y]	0.61 ± 0.18	0.58 ± 0.14
5	0.49 ± 0.05	0.44 ± 0.04	ND	46.67 ± 1.52	52.20 ± 1.16	0.37 ± 0.06	ND	ND
10	0.50 ± 0.06	0.46 ± 0.04	ND	46.93 ± 1.03	52.08 ± 1.92	ND	ND	ND

[x,y]Means within CLA oil supplement and a wk 1 storage product with different superscripts differ ($P < 0.05$); differences by industrial treatment.

[a,b]Means within a product and column with different superscripts differ ($P < 0.05$); differences by refrigeration storage.

[1]c = *cis*; t = *trans*; ND = not determined.

The CLA isomer compositions (% of total CLA) of the 6 CLA-enriched products studied after 1, 5, and 10 wk of storage are shown in Table 3. To determine the possible variations in CLA isomer composition during the processing of the CLA-enriched products, CLA-supplemented oil was used as a control during the first week of storage of the different products. Silver ion-HPLC identification of more than 10 different peaks attributed to CLA isomers was based on coinjection with reference material because of retention time irreproducibility and in accordance with the elution order, as we reported in previous studies with the same chromatographic conditions (Luna et al., 2005a). The Ag^+-HPLC profile of lipid sample was shown to separate the different C18:2 *trans*, *trans* compounds followed by a chromatographic zone where *cis*, *trans* and *trans*, *cis* isomers were located and finally the *cis*, *cis* CLA isomers eluted. The most prominent peaks in all products corresponded, as expected, to C18:2 *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers, which accounted for about 95% of total CLA. All enriched milk products showed a similar ratio of C18:2 *cis*-9, *trans*-11 to *trans*-10, *cis*-12 CLA, which ranged from 0.95 to 0.98.

The CLA isomer percentages of the enriched products studied differed substantially from proportions reported for total CLA fatty acids in cow's milk fat, in which most of the CLA corresponds to RA (about 80%) and which would be a poor source of the C18:2 *trans*-10, *cis*-12 CLA isomer (Sehat et al., 1998). Similarly, the C18:2 *trans*-7, *cis*-9 CLA isomer is the second greatest in cow's milk fat (5 to 10% of total CLA), whereas its presence in the products of the current study seemed to be negligible.

Noticeable percentages of the isomers C18:2 *cis*-11, *trans*-13 and *trans*-8, *cis*-10 were found in the fortified products (1.8 and 1.7%, respectively, in the CLA-supplemented oil and up to 2.4% in CLA-enriched cheese). Amounts of 4.2% of C18:2 *cis*-11, *trans*-13 and lower than 1% of C18:2 *trans*-8, *cis*-10 of total CLA were reported by Luna et al. (2005a) in ewe's milk.

Another 4 peaks eluting in the C18:2 *trans*, *trans* CLA region were assigned to *trans*-9, *trans*-11; *trans*-10, *trans*-12; *trans*-11, *trans*-13; and *trans*-12, *trans*-14. Two of these isomers (*trans*-9, *trans*-11 and *trans*-12, *trans*-14) were in trace amounts in the CLA oil supplement and not detectable in the analyzed samples, whereas the 2 peaks identified as *trans*-10, *trans*-12 and *trans*-11, *trans*-13 accounted for 0.5 and 0.6% of the CLA-oil supplement, respectively.

In the C18:2 *cis*, *cis* CLA chromatographic area, 2 isomers with comparable amounts (0.6%) were detected in the CLA oil and assigned to *cis*-9, *cis*-11 and *cis*-10, *cis*-12. These isomers were not detected in different dairy foods when CLA profiles were researched using

Ag^+-HPLC in similar conditions (Sehat et al., 1999; Luna et al., 2005a).

Effects of Processing and Storage on CLA Isomers by Ag^+-HPLC

The important quality issues for CLA-supplemented products are total CLA content and isomeric distribution that could be altered by the effect of processing conditions, storage, and aging.

To determine significant variations in CLA isomer composition during processing of the CLA-enriched products, CLA-supplemented oil was used as a control in the first week of storage of the different products. The CLA isomer contents as a percentage of total CLA are shown in Table 3. No significant changes were found in the content of the major isomers (C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 CLA) as a consequence of treatment or refrigerated storage up to 10 wk in any of the CLA-enriched dairy products studied. Nevertheless, this study showed that the industrial process of the product or thermal treatment resulted in a significant decrease of some of the minor CLA isomers such as *cis*-11, *trans*-13 and *trans*-8, *cis*-10 in milk powder, fermented milk, yogurt, and milk-juice blend.

In the CLA-enriched fresh cheese studied, the formation of new isomers was not found, but a significant decrease of the minor isomer C18:2 *cis*-11, *trans*-13 during refrigerated storage, and even the disappearance of some other C18:2 *cis*, *cis* isomers as *cis*-9, *cis*-11 and *cis*-10, *cis*-12, was observed. Decreases in these minor isomers resulted in undetectable levels in other samples, such as the milk-juice blend.

Isomer contents in the C18:2 *trans*, *trans* region (mainly *trans*-9, *trans*-11 and *trans*-10, *trans*-12 isomers) were found to be low in all CLA-enriched products except for milk powder, in which levels increased significantly after the thermal process. The level of C18:2 *trans*, *trans* isomers was also positively influenced in milk after 5 wk of storage but not significantly. The increase in the content of C18:2 *trans*, *trans* isomers has been related to lipid transformation by heat treatment (Precht et al., 1999; Juanéda et al., 2003; Herzallah et al., 2005). It has been reported that the *trans* double bond is more stable compared with the *cis* bond in the same environmental conditions, and that the level of *trans/trans*-CLA may be elevated by reaction conditions that favor thermodynamic products such as a higher reaction temperature and longer reaction time (Bruice and Lightstone, 1999). Destaillats et al. (2005) found that severe thermal processes result in sigmatropic isomerization of RA resulting in the formation of C18:2 *trans*-8, *cis*-10, which can be used as a marker of heat treatment of natural fats and oils containing CLA. Sigmatropic rearrangement has been also described between the isomers C18:2 *trans*-10, *cis*-12 and *cis*-11, *trans*-13, and between C18:2 *cis*-9, *trans*-11 and *trans*-8, *cis*-10, with a significant increase of C18:2 *trans*, *trans* isomers from *cis*, *trans*; *trans*, *cis*; and *cis*, *cis* isomers.

In summary, this study indicates that the total CLA content of different commercially available CLA-enriched dairy products (supplemented using Tonalin-80, an oil supplement with 80% CLA) varied considerably from 50 to 75% depending on the presence of milk fat in the products. The CLA isomers C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 were the predominant fatty acids present in all products, at a ratio ranging from 0.97 to 1.05. These major isomers were not affected by the processing used and did not significantly decrease after 10 wk of storage. Only a significant loss of total CLA throughout the refrigerated storage of fresh cheese sample was found, possibly related to an increase in microbiota growth. Nevertheless, refrigerated storage and, particularly, thermal treatment resulted in significant decreases or disappearance of some of the minor CLA isomers and a significant increase of C18:2 *trans*, *trans* isomers from *cis*, *trans*; *trans*, *cis*; and *cis*, *cis* isomers especially in CLA-fortified milk powder but also in fermented milk, yogurt, and milk-juice blend.

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DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL.

Los resultados obtenidos en el presente trabajo se discuten de acuerdo a los cuatro objetivos fijados:

4.1 Desarrollo, optimización y validación de métodos cromatográficos para el estudio de la fracción lipídica de productos lácteos.

La grasa láctea presenta una composición compleja compuesta principalmente por triglicéridos (97-98%) con cerca de 400 ácidos grasos diferentes, de 4 a 26 átomos de carbono, aunque solo un número próximo a 30 está en una proporción superior al 0,1%. Además, cabe citar la presencia de ácidos grasos de cadena ramificada, isómeros posicionales o geométricos, etc. Estos ácidos grasos presentan una gran variabilidad, ya que como es conocido, su composición puede sufrir importantes variaciones como consecuencia de la especie, raza, dieta y estado de lactación del ganado. Otros componentes lipídicos como diglicéridos, fosfolípidos, colesterol se encuentran entre el 0,3-1 % y el resto, como monoglicéridos, ácidos grasos libres, etc., en concentraciones menores, por lo que el análisis de cada compuesto requiere de técnicas de análisis específicas o de la combinación de estas. La determinación cuantitativa y cualitativa de muchos de estos componentes ha alcanzado en la actualidad una gran relevancia dado que muchos de ellos han sido relacionados con efectos beneficiosos o perjudiciales para la salud del consumidor, como son el ácido linoleico conjugado o los ácidos grasos *trans*.

4.1.1 Optimización de un método rápido por cromatografía de gases para el análisis rutinario de la composición en ácidos grasos de grasas y aceites alimentarios.

Los resultados aquí discutidos corresponden con el capítulo: "Analysis of milk fat, vegetable and fish oil fatty acids using a short time GLC method".

La cromatografía de gases es la principal técnica empleada en el análisis cualitativo y cuantitativo de los ácidos grasos presentes en una grasa o aceite alimentario. Sin embargo dichos análisis precisan etapas previas de preparación de la muestra en la que los ácidos grasos son derivatizados en compuestos de mayor volatilidad como son los ésteres metílicos (FAMEs). Dada la complejidad de la grasa láctea, para el análisis de FAMEs se han empleado columnas capilares con fases polares de elevada selectividad (como ciano-propilo) y longitud (de 50 a 130 m) para obtener la mejor resolución posible entre los diferentes componentes presentes (ej. diferenciación entre isómeros cis y *trans*). En el laboratorio en donde se ha desarrollado el presente trabajo, se disponía de metodologías de análisis por GC-FID mediante el empleo de la columna CPSil-88 de 100 m de longitud (Varian), con la que se puede obtener una descripción completa de la composición en ácidos grasos de grasa láctea, dada la alta capacidad resolutiva de esta columna. No obstante, los tiempos de análisis empleados para cada muestra son también muy largos, en el caso concreto de esta columna se alcanzan los 105 minutos, por lo que para llevar a cabo un análisis rutinario con muchas muestras se hacía necesaria la optimización del método pero sin influir en gran medida en la resolución. Con este objetivo, se empleó la columna VF23ms con la misma fase pero de 30m x 0.25mm x 0.25µm (Varian) y dos programas de temperaturas. Uno de ellos (P0) fue desarrollado para el análisis de grasa láctea en donde se persigue una buena elución y resolución de los ácidos grasos de cadena corta, compuestos *trans* y del linoleico conjugado CLA. Para la puesta a punto del procedimiento se empleó una grasa láctea de referencia CRM164 (Fedelco, Madrid). Igualmente se validó el procedimiento para el estudio de la composición de distintos aceites vegetales y de pescado mediante optimización del programa de temperatura empleado (P1).

Los análisis de la grasa de referencia CRM164 muestran que la columna CPSil-88 consigue la separación de los ácidos grasos C18:1 *trans* en 5 picos distintos (C18:1 t6-t8, C18:1 t9, C18:1 t10, C18:1 t11, C18:1 t12-14t) pudiéndose observar la presencia traza de C18:1 t4 y C18:1 t5. También es posible

diferenciar los isómeros cis minoritarios del ácido oleico (C18:1 cis 9) como los compuestos C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14 (que coeluye con el t16) y el C18:1 c15. De los isómeros minoritarios de ácido linoleico (C18:2 c9,c12) pueden detectarse la presencia de los C18:2 t,t NMID (dobles enlaces no interrumpidos por metileno), C18:2 t9,t12, C18:2 c9,t12, C18:2 t11,c15, C18:2 t9,c12 y C18:2 t9,c15. En cuanto a los isómeros de CLA, además de la presencia de ácido ruménico (C18:2 c9,t11) con esta columna y nuestras condiciones de trabajo es posible la cuantificación de C18:2 t8,10c, C18:2 t11,13c, C18:2 t10,12c, C18:2 9c,11c, C18:2 10c,12c, C18:2 11c,13c y C18:2 *trans,trans (total)*. Se determinan también los isómeros de CLnA (uno de ellos identificado como C18:3 c9,t11,t13) en ocasiones presentes en grasa láctea. Como se ha indicado anteriormente, el tiempo total de análisis con esta columna es de 105 minutos.

En el análisis de FAMEs de aceites vegetales y de pescado mediante la aplicación de esta columna, es posible igualmente determinar, además de los ácidos de longitud de cadena media, un número muy elevado de ácidos grasos de larga y muy larga longitud de cadena como EPA, DPA y DHA, con el mismo programa utilizado anteriormente para grasa láctea.

El empleo de la columna VF23 ms con el programa de temperatura P0 para el análisis de CRM164 revela, que los compuestos *trans* del oleico son resueltos en tres picos C18:1 t4-t9, C18:1 t10 y C18:1 t11 (TVA), mientras que los compuestos C18:1 *cis* aparecen en el orden C18:1 c11, C18:1 c12 y C18:1 c13+c14. La elución para los compuestos del C18:2 fue *trans,trans; cis,trans/ trans,cis y cis,cis*. Mediante el empleo del programa de temperaturas P1 para el análisis de aceites vegetales y de pescado pudo observarse que es posible diferenciar los isómeros C18:2 t,t NMID, C18:2 t9,t12, C18:2 c9,t13, C18:2 c9,t12 y C18:2 t11,c15, puesto que en estas condiciones todos estos ácidos grasos tuvieron tiempos de retención distintos. En estas condiciones también fue posible diferenciar los isómeros de CLA: RA, C18:2 t10,c12, C18:2 c,c (total) y C18:2 t,t (total). Igualmente se determinan isómeros de CLnA además de AA, EPA, DHA, DPA.

Los resultados obtenidos muestran composiciones y contenidos similares a otros previamente reportados para grasa láctea (Jensen, 2002), girasol, cártamo y soja (Dubois *et al.*, 2007), tonalin® (Rodriguez-Alcala & Fontecha, 2007), tung (Cao *et al.*, 2007) y pescado (Fournier *et al.*, 2006). Los valores CV calculados para cada método muestran una buena repetitividad y reproducibilidad de los análisis llevados cabo con cada columna y los resultados son comparables entre sí al existir diferencias inferiores al 10% (Horwitz, 1982). Además, como ventaja significativa, indicar que el tiempo necesario para la elución de todos los picos presentes en grasa láctea, aceites vegetales y de pescado fue inferior a 17 min.

Diversos trabajos previos ponen de manifiesto la dificultad de reducir el tiempo de análisis sin comprometer la resolución de los diferentes ácidos grasos. En trabajos llevados a cabo con columnas de 10 metros, con hidrogeno como gas portador, el análisis de grasa láctea se puede obtener en 4 minutos. Sin embargo los C18:1 *trans* y *cis* no son separados y coeluyen en un único pico, mientras que de los isómeros de CLA, solamente se detecta RA (Destaillats & Cruz-Hernandez, 2007). En otros estudios empleando columnas de mayor longitud (40m) y Helio como gas portador, muestran que en el análisis de mezclas de patrones y grasa láctea procedente de leche humana, el ácido C18:1 *trans* y *cis*, C18:2 *trans*, *trans* y C18:2 cis, *trans-trans, cis* eluyen cada uno en un pico, no pudiéndose obtener una composición más detallada, mientras que para los compuestos de CLA es posible la cuantificación de RA, C18:2 c11,t13, C18:2 t10,c12 y C18:2 t,t CLA (Bondia-Pons *et al.*, 2007; Moltó-Puigmarti *et al.*, 2007). Otros estudios de cromatografía rápida, con columnas de 30 metros, muestran que en el análisis de mezclas de patrones cromatográficos, los isómeros de los ácidos grasos *cis* y *trans* de C18:1 se solapan y no pueden diferenciarse y que para los del C18:2, aunque pueden separarse *trans-trans* de *cis-cis*, los *cis,trans-trans,cis* coeluyen, siendo la situación similar para los compuestos de CLA.

Con las condiciones analíticas descritas en el presente trabajo se ha conseguido realizar un análisis rápido de la composición de FAMEs tanto de grasa láctea como de diferentes aceites comestibles mostrando una buena

resolución y reproducibilidad para los compuestos minoritarios e isómeros de los principales componentes como oleico, linoleico, linolénico y CLA. Es posible por tanto su utilización como método de rutina para el estudio del perfil de ácidos grasos de un número elevado de muestras.

4.1.2 Validación de un método de análisis mediante HPLC-ELSD, de las distintas clases lipídicas presentes en grasa láctea, con especial interés en la fracción de fosfolípidos.

Los resultados aquí discutidos corresponden con en el capítulo:" Major lipid classes separation of buttermilk, and cows, goats and ewes milk by HPLC-ELSD focused on the phospholipid fraction".

El análisis de los distintos componentes de la grasa láctea tiene en la gran diversidad de su composición, su principal dificultad. Ello hace necesario el empleo de técnicas de fraccionamiento y concentración (ej. TLC, SPE, etc.) previas al análisis cromatográfico, de tal forma que no se produzcan coeluciones entre los distintos componentes, fundamentalmente de los triglicéridos que son la fracción mayoritaria. Trabajos previos describen el análisis de fosfolípidos en distintos productos lácteos mediante HPLC-ELSD (Rombaut *et al.*, 2005; Lopez *et al.*, 2008), pero sin separar los lípidos neutros de la grasa láctea (tri, di y monoglicéridos, colesterol y ácidos grasos libres) que coeluían en un mismo pico y saturaban el detector. Otros trabajos optaron por el empleo de cromatografía de gases, previo aislamiento de los fosfolípidos (Bondia-Pons *et al.*, 2006).

El objetivo del presente trabajo consiste en la separación cualitativa y cuantitativa de las distintas fracciones lipídicas presentes en grasa láctea, con especial interés en el contenido en fosfolípidos, pero sin fraccionamiento previo y en una sola carrera cromatográfica

Mediante el empleo de patrones puros de compuestos presentes en las distintas clases lipídicas y de fosfolípidos, se ajustó el gradiente de disolventes

para obtener un perfil cromatográfico cuyo orden de elución fue: ésteres de colesterol, TG, colesterol, DG y FFA (estos tres últimos en un mismo pico), moglicéridos (MG), fosfatidiletanolamina (PE), fosfatidilinositol (PI), fosfatidilserina (PS), fosfatidilcolina (PC), esfingomielinas (SM) y lisofosfatidilcolina (LPC). Estudios previos realizados en sistemas similares describen la separación de TG, FFA, MG, 1,2-DG y 1,3-DG, aunque no muestra la separación de fosfolípidos (Torres *et al.*, 2005). Otras investigaciones desarrolladas para llevar a cabo el análisis de TG, DG, MG y fosfolípidos en suero humano consiguen también separar los distintos compuestos aunque los fosfolípidos coeluyen en un mismo pico y no es posible realizar un análisis de las diferentes clases (Perona & Ruiz-Gutierrez, 2004).

Para determinar la respuesta del detector para los distintos fosfolípidos, en el presente método se inyectaron concentraciones crecientes de PE, PS, PI, PC, SM y LPC. Los datos obtenidos permitieron ajustar la respuesta del detector a las ecuaciones obtenidas al aplicar el modelo cuadrático potencial, lo cual coincide con lo reportado en los trabajos previos ya comentados y otros publicados recientemente (Ramos *et al.*, 2008).

La viabilidad del método fue estudiada mediante el análisis de grasa láctea procedente de mazadas en polvo (producto de suero de quesería rico en fosfolípidos de las membranas de glóbulo graso) que fueron aisladas por duplicado mediante el método de Folch (Folch *et al.*, 1957) para asegurar la extracción completa de los diferentes compuestos de interés, puesto que otros autores han descrito que otras metodologías producen extracciones selectivas de los fosfolípidos (Avalli & Contarini, 2005). Los resultados obtenidos mostraron que como era de esperar los compuestos mayoritarios fueron los triglicéridos, seguidos de colesterol, DG y FFA y en concentraciones similares, MG y ésteres de colesterol. En la fracción de fosfolípidos, que constituyó el 30 % del total de lípidos, PC fue el compuesto mayoritario, seguido de PE, PS y SM que mostraron concentraciones similares entre si. Aunque existe una gran variabilidad en cuanto al contenido reportado para el total de fosfolípidos presente en mazadas (4.5-33%) (Rombaut *et al.*, 2006; Rombaut & Dewettinck, 2006), los datos y distribuciones aquí presentados coinciden con los de otras

investigaciones previas (Miura *et al.*, 2004; Britten *et al.*, 2008). En todos los ensayos los resultados mostraron coeficientes de variación inferiores al 10%, lo que indica que las condiciones aplicadas fueron adecuadas para el análisis realizado (Horwitz, 1982).

El análisis de fosfolípidos en grasa de leche de cabra, vaca y oveja, mostró que el contenido de estos compuestos es mayor en cabra que en vaca y oveja, coincidiendo con resultados previamente publicados para leche de rumiantes (Contarini *et al.*, 2009). Otros sin embargo describen concentraciones similares entre diferentes especies de mamíferos (Lamothe *et al.*, 2008), aunque las diferencias pueden atribuirse a la genética, estado de lactación y alimentación de los animales (Chilliard *et al.*, 2007; Lopez *et al.*, 2008).

Por tanto, respecto a los trabajos comentados, el presente método cromatográfico supone una mejora en la detección y cuantificación de clases lipídicas de la grasa láctea, sin necesidad de fraccionamiento previo o concentración de la muestra. Además, permite determinar cualitativa y cuantitativamente el contenido en fosfolípidos de las distintas muestras con una elevada resolución, lo que adquiere un gran interés debido a las propiedades bioactivas descritas para estos componentes.

4.2 Estudio de la mejora nutricional del perfil lipídico de la leche mediante la incorporación a la dieta de rumiantes de suplementos ricos en PUFA, o mediante empleo de bacterias lácticas con capacidad para transformar el ácido linoleico en isómeros del ácido linoleico conjugado (CLA).

4.2.1 Efectos de la suplementación de la dieta de cabras con semillas de lino en la composición de la grasa de la leche.

Los resultados aquí discutidos corresponden con el capítulo: "Influence of feeding linseed at different levels on fatty acid profile focused on the CLA isomers composition of goat milk".

Entre las leches de consumo humano, la leche de cabra y oveja presentan un gran interés nutricional, ya que desde el punto de vista lipídico su composición se caracteriza por contener concentraciones muy superiores en ácidos grasos de cadena corta y media que la de vaca. Estos ácidos grasos son fácilmente asimilados por nuestro organismo ya que forman parte de TG de bajo peso molecular que al ser hidrolizados por las lipasas digestivas del estómago no precisan reesterificación para ser reabsorbidos, mientras que en el resto de triglicéridos, la hidrólisis es terminada por la lipasa pancreática. Estas leches, pero especialmente la de cabra son por ello especialmente indicadas en situaciones de desnutrición o de insuficiencia pancreática. Además, la explotación de estos animales se desarrolla en zonas áridas o semiáridas y son respetuosas con el medio ambiente. La grasa de estas leches contienen además un mayor porcentaje en ácidos grasos TVA y CLA, aunque la presencia de ácidos grasos saturados es igualmente elevada. Es por ello que, al igual que en leche de vaca, existe un gran interés en mejorar la calidad nutricional de la leche de estos rumiantes mediante la suplementación de la dieta con PUFAs y de esta manera incrementar la composición en ácidos grasos bioactivos.

Para esta investigación tres explotaciones de cabras fueron seleccionadas para ser alimentadas con dietas suplementadas con semillas de lino a tres niveles o dosis de suplemento (0.3, 0.5 y 0.7 kg suplemento/animal/día) y los resultados

fueron comparados con aquellos obtenidos de animales alimentados sin suplemento. Leche de las tres explotaciones suplementadas y control fueron analizadas en su composición global y su fracción grasa para determinar la mejora en la composición en ácidos grasos de la misma. Los resultados obtenidos del análisis cromatográfico mostraron una disminución significativa de los ácidos grasos saturados principalmente de ácido palmítico, mirístico y láurico, aunque las concentraciones de los ácidos grasos de cadena corta y media no fueron modificadas por efecto de la suplementación. Estos resultados pueden atribuirse, al igual que sucede en vacas suplementadas con lino, a una inhibición de la síntesis *de novo* o a un efecto de dilución por el incremento significativo en PUFAs. Igualmente las concentraciones de esteárico, oleico, TVA, LA y CLA (principalmente RA y C18:2 t11,t13) se incrementaron significativamente hasta prácticamente duplicar su valor inicial para el CLA. Así mismo, indicar que se ha conseguido identificar los distintos isómeros de CLA mediante el desarrollo y combinación de diversos procedimientos cromatográficos (GC-FID/GC-MS/HPLC-Ag+).

Especialmente reseñable son los incrementos producidos en el contenido de ácido α-linolénico (omega-3) y TVA hasta valores próximos o superiores al triple de su nivel. Al contrario que lo observado en estudios con leche de vaca, el contenido en C18:1 t10 no se modificó por la suplementación, lo que implicaría que no se produce una alteración del entorno del rumen, o al menos no en la misma extensión/forma que en vaca.

Actualmente está ampliamente aceptado que, al igual que en el ganado bovino, los mejores resultados al suplementar la dieta de cabras y ovejas, se obtienen al emplear fuentes ricas en linolénico como son las semillas de lino, o linoleico como el girasol o soja así como sus correspondientes aceites. En experiencias previas donde se alimentaron cabras con semillas de lino (11%) y semillas de lino tratadas con formaldehido (11%) o extrusionadas (16%) para mejorar la biohidrogenación de los PUFA en el rumen (Bernard *et al.*, 2005; Nudda *et al.*, 2006), mostraron resultados similares a los obtenidos en este estudio, con mayores variaciones para palmítico, esteárico y oleico, pero con menores incrementos de CLA (177% vs. 106%).

En los resultados presentados se puede observar que los mejores resultados en la relación SAT/PUFA se obtuvieron en la leche de la explotación que recibió mayor cantidad de suplemento (0.7 Kg). No obstante, estos valores no fueron significativamente superiores a los encontrados para el grupo suplementado con una cantidad intermedia (0.5 Kg), dado que tampoco pueden descartarse otros factores implicados en la composición de la grasa láctea, como son la genética del animal, su estado de lactación, etc. En un estudio posterior, que no figura en esta memoria, se han realizado quesos de cabra a partir de las leches obtenidas de las explotaciones suplementadas. Los resultados obtenidos en el contenido en ácidos grasos, mostraron los mismos resultados a nivel cuantitativo que los expuestos en el presente trabajo. Indicar además que no se encontraron diferencias significativas entre los quesos control y los elaborados con leche suplementada en cuanto a sus características sensoriales.

4.2.2 Estudio de la capacidad de bacterias lácticas y bifidobacterias para la transformación de ácido linoleico en isómeros del ácido linoleico conjugado (CLA).

Los resultados aquí discutidos corresponden con el capítulo: "Quantitative and qualitative determination of CLA produced by Bifidobacterium and LAB combining spectrophotometric and Ag+-HPLC techniques".

Como se ha indicado, son las bacterias *Butyrivibrio fibrisolvens* presentes en el rumen las responsables de la transformación de los ácidos grasos insaturados de la dieta del rumiante. Este hecho llevó a plantearse si otras bacterias lácticas (LAB) o bifidobacterias, empleadas en la fermentación de la leche, serían capaces de, en presencia de distintos PUFAs como sustrato, producir isómeros de CLA.

Para el estudio se contó con un total de 22 bacterias (16 *Lactobacillus*, 5 *Bifidobacterium* y 1 *Lactococcus*), por lo que en un primer ensayo se trató de identificar aquellas que mostraran dicha capacidad tras llevar a cabo la

reacción en medio de cultivo MRS. Para la detección de CLA se desarrolló un procedimiento combinado de espectrofotometría UV y HPLC-Ag^+, lo cual permitiría determinar tanto las concentraciones producidas como los isómeros presentes.

En esta primera etapa del estudio, 6 bacterias mostraron capacidad para transformar linoleico en CLA (2 *Bifidobacterium*, 2 *Lactobacillus* y 2 *Lactococcus*). Comparando estos resultados con los reportados en las revisiones de Sieber *et al.*(2004) y Ogawa *et al.* (2005), se observa que la producción obtenida de CLA en este estudio fue ligeramente inferior en todos los casos excepto para uno de los bifidobacterium. Ello puede deberse a características propias de los microorganismos, además de que los PUFAs ejercen actividades antimicrobiológicas (Nieman, 1954) por lo que algunos autores sostienen que la transformación en CLA no sería sino un mecanismo de detoxificación (Adamczak et al., 2008).

Tras la identificación y aislamiento, las bacterias seleccionadas fueron ensayadas en leche desnatada, empleando como sustrato añadido o bien ácido linoleico (LA) en su forma libre o aceite de cártamo (con un 80% de contenido en LA), evaluando la producción de CLA a las 24h y las 48h de iniciada la incubación. En estas condiciones, aunque los microorganismos mostraron capacidad de producir CLA a partir de ambos substratos, se determinó que en el caso de LA libre, 24h era el tiempo adecuado de reacción, al presentar mayor producción de CLA. Con el empleo de aceite de cártamo como sustrato, las mayores concentraciones de CLA se observaron a las 24h pero el hecho de que para acceder al LA, los microorganismos deben producir lipasas y esterasas (Holland *et al.*, 2005) hizo que se seleccionaran 48h como tiempo óptimo de reacción. En algunos casos se observó que la concentración de CLA fue menor a las 48h, lo que se explica en otros estudios con resultados similares, como una consecuencia de la degradación oxidativa del CLA por parte de los microorganismos (Wang *et al.*, 2007).

Finalmente, nuevos ensayos con las bacterias seleccionadas en leche desnatada y en las condiciones descritas, permitieron confirmar los resultados previos obtenidos y que ponen de manifiesto la producción de CLA por parte de

las bacterias en mayor concentración cuando se emplea LA libre como substrato. Los isómeros producidos fueron identificados como C18:2 c9, t11 (RA), C18:2 t10, c12 y sus formas *trans, trans*, lo que se corresponde con los isómeros de CLA presentes en aceites comerciales de alto contenido en CLA obtenidos por síntesis química (Ma *et al.*, 1999). Estos resultados coinciden con otros previamente publicados por otros autores en experiencias similares donde bacterias del género *Lactobacillus*, *Lactococcus* y *Bifidobacterium* mostraron la capacidad de transformar LA en los isómeros de CLA, RA, C18:2 t10,c12 y C18:2 t9,t10 en leche desnatada y medios de cultivo respectivamente (Alonso *et al.*, 2003; Coakley *et al.*, 2003).

Las concentraciones de CLA encontradas en leche desnatada en el presente trabajo se encuentran en el rango de 2.72-3.44 mg CLA/g grasa cuando se emplea LA como substrato y 0.31-1.53 mg CLA/g grasa con cártamo. Estas concentraciones son inferiores a las reportadas en otros estudios previos en donde *L. brevis,* aislado de fluidos ruminales producía 10 mg CLA/g grasa, en leche desnatada usando 0.25 % de aceite de girasol como fuente de LA, mientras que *L. lactis* con 1% de aceite de girasol produjo 9.22 mg CLA/g grasa (Puniya *et al.*, 2008). Otros trabajos han descrito como *L. acidophilus, L. casei* y *L. Lactis* incubados en leche de búfala pasteurizada, producían 6 mg CLA/g grasa a partir de grasa láctea (Yadav *et al.*, 2007).

Aunque el presente estudio ha permitido establecer las condiciones analíticas para cuantificar el contenido de CLA y los medios para seleccionar los microorganismos productores de CLA, todavía es necesario seguir profundizando en este campo de investigación para alcanzar la optimización de estos procedimientos en futuros estudios que permitan una mayor producción de CLA por microorganismos en un medio lácteo.

4.3 Evaluación del efecto de los tratamientos industriales convencionales (pasterización, UHT y esterilización) frente a los no convencionales (microondas, altas presiones y homogenización a alta presión) sobre el perfil lipídico y el contenido en fosfolípidos de leche de vaca, oveja y cabra.

4.3.1 Procesado convencional (pasterización, UHT y esterilización) y no convencional (microondas y altas presiones) aplicado a leches de vaca.

Los resultados aquí discutidos en la sección siguiente corresponden con el capítulo: "Influence of heat treatments, high pressure and microwave processing of naturally PUFA enriched milk on CLA isomers distribution and trans fatty acids content".

Para determinar los posibles efectos de los tratamientos tecnológicos (térmicos, altas presiones y microondas) sobre la composición de los ácidos grasos de leche de vaca y si se ven afectados por el grado de insaturación de la grasa láctea, se obtuvieron dos lotes de leche de vaca. Un lote de 500 litros de leche procedente de vacas alimentadas con una dieta suplementada con semillas ricas en PUFA (lino y maíz) (leche de alto contenido en insaturados) y otro lote con una alimentación sin suplemento (leche control). Ambos lotes fueron procesados por los mismos tratamiento térmicos (pasterización, HTST, UHT y esterilización) y no convencionales (microondas y altas presiones).

Se registraron cambios en la fracción de CLA, como consecuencia del proceso térmico de esterilización y concretamente en el contenido del isómero C18:2 t9,t11 cuyo contenido aumentó en ambos lotes ($p<0,05$). También se registró un incremento significativo de CLA (C18:c9t11, RA) ($p<0,05$) en la leche de alto contenido en insaturados, cuando fue sometida a alta pasterización (HTST) y microondas, frente a leche control que mostró un aumento no significativo. Herzallah *et al.* (2005) en leche tratada mediante pasterización y microondas en condiciones similares a las de este trabajo, encontraron que el contenido total de CLA disminuía tras estos tratamientos. Campbell *et al.* (2003) en leche

enriquecida con aceites de alto contenido en CLA, no registraron alteraciones como consecuencia de la pasterización, al igual que otros estudios (Lynch *et al.*, 2005) también en leche pasterizada de alto contenido en TVA y PUFAs (por la alimentación de las vacas). Estudios previos del efecto de procesado a altas temperaturas a mantequilla, concluían que la temperatura podía provocar la isomerización de los dobles enlaces *trans* al haberse relacionado con disminuciones de oleico y con incrementos en elaídico (Precht *et al.*, 1999). Otros trabajos han demostrado que las altas temperaturas pueden producir la isomerización de ácido linoleico en los isómeros C18:2 t10, t12 y C18:2 t9,t11 en grasa láctea mediante reacciones de reordenamiento sigmatrópico, alterando la distribución de los isómeros de CLA (Destaillats & Angers, 2005; Destaillats *et al.*, 2005).

Los resultados del presente estudio indican que el incremento encontrado del isómero C18:2 t9, t11, sería debido a una isomerización del LA por efecto del proceso de esterilización de la leche, independientemente del contenido en PUFAs. En leche pasterizada HTST y tratada con microondas, el aumento en RA pudiera deberse a reacciones de reordenamiento sigmatrópico, si bien no se ha observado una relación entre este aumento y variaciones de otros isómeros de CLA. Los tratamientos por altas presiones no producen modificaciones significativas en el perfil de ácidos grasos de leche.

4.3.2 Tratamiento por homogenización a altas presiones (HPH) aplicado a leches de vaca, oveja y cabra.

Los resultados aquí discutidos en la sección siguiente corresponden con el capítulo: "Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization".

Leche cruda de vaca, oveja y cabra fue sometida a procesos HPH en un rango de presiones de 50 a 350 MPa. El primer aspecto importante observado, fue que la cantidad de grasa extraída mediante procesos de centrifugación, era inversamente proporcional a la presión aplicada en el tratamiento. Así, en

muestras de leche de oveja, no fue posible llevar a cabo el aislamiento de la fracción grasa en tratamientos por encima de los 250 MPa. Estos procesos parecen estar relacionados con un incremento de la viscosidad y la dispersión de la grasa contenida en el glóbulo graso, también reportado en otros estudios llevados a cabo en leche de vaca como la formación de agregados grasos a presiones superiores a los 300 MPa (Pereda *et al.*, 2007). Igualmente que en altas presiones hidrostáticas, en un rango de 100-800 MPa, se ha reportado la asociación de proteínas de suero con la membrana del glóbulo graso (MFGM) en leche entera (Ye *et al.*, 2004).

Estas posibles alteraciones de la estructura del glóbulo graso y las asociaciones de la MFGM con proteínas por efecto de la presión, podrían hacer suponer que a presiones de 300 MPa o superiores, se observarían cambios en los contenidos de los ácidos grasos. Este aspecto se considera de relevancia si se tiene en cuenta que las altas presiones promueven la desnaturalización parcial de las proteínas del suero y de la MFGM, aún cuando estas no forman agregados entre sí (Hayes *et al.*, 2005; Roach & Harte, 2008). Además, se ha descrito que los tratamientos HPH inferiores a 200 MPa no inactivan las posibles lipasas microbianas presentes en la leche, conduciendo a un incremento en la concentración de ácidos grasos libres (Datta *et al.*, 2005; Pereda *et al.*, 2008). El estudio en profundidad de la composición en ácidos grasos e isómeros de CLA, realizado en este trabajo de investigación, para leches de vaca, oveja y cabra, tratadas mediante HPH (50-350 MPa), y comparadas con leche control, no permitió determinar variaciones significativas en los contenidos en ácidos grasos.

4.3.3 Tratamiento a muy altas presiones de hasta 900 MPa aplicado a leche vaca.

Los resultados aquí discutidos en la sección siguiente corresponden con el capítulo: "Cow milk processed at very high pressure: effects on the fatty acids and phospholipids composition".

Para el presente estudio se emplearon leches de vaca recogidas en Abril y Junio y tratadas por altas presiones en un rango de 250-900 MPa. Son muy escasos los estudios en la bibliografía que aplican altas presiones hasta 900 MPa, y mucho menos los interesados en conocer sus efectos sobre la fracción lipídica de la leche.

En relación con la composición de ácidos grasos, las muestras analizadas se corresponden con otras previamente reportadas (Jensen, 2002) y se observó que la composición de la leche obtenida en Junio mostraba un perfil graso más saturado ($p<0.05$) como consecuencia de la estacionalidad y posiblemente del estado de lactación de los animales (Bauman & Griinari, 2003; Chilliard *et al.*, 2007).

En la distribución y contenidos hallados para la fracción de fosfolípidos, los componentes mayoritarios fueron fosfatidiletanolamina (PE), fosfatidilcolina (PC) y esfingomielinas (SP) lo que coincide con otros trabajos previamente publicados (Jensen, 2002; Avalli & Contarini, 2005; Rombaut *et al.*, 2007). De nuevo se observaron diferencias significativas en los contenidos de PE, PI, PS y SM que fueron mayores en las muestras de leche obtenidas en Abril mientras que el contenido en PC fue mayor en las obtenidas en Junio. Previamente se ha descrito que la composición en fosfolípidos en leche de rumiantes está influenciada por la genética, alimentación, estado de lactación etc (Chilliard *et al.*, 2007; Lopez *et al.*, 2008).

En cuanto al efecto por el procesado no se observaron variaciones en el perfil de ácidos grasos a ninguna de las presiones ensayadas y no fueron afectadas por las diferencias en cuanto a contenidos, lo que coincide con los resultados obtenidos en estudios previos. Sin embargo, el estudio de la fracción de fosfolípidos (PLs) reveló que en el rango de presiones de 250-800 MPa se producían alteraciones en la distribución de estos componentes al disminuir las concentraciones de PE, PS y PI en las muestras procedentes de leche recogida en Abril, mientras que solamente PI y PS mostraron este comportamiento en las muestras tratadas con la leche recogida en Junio.

Estos PLs se encuentran en la lámina interna de la membrana del glóbulo de grasa (MFGM), por lo que las variaciones observadas, sugieren que durante el

procesado se produce una disrupción del glóbulo graso. Diversos autores han sugerido que los procesos de homogenización y otros, empleados en la elaboración de productos lácteos, así como su almacenamiento, alteran la MFGM debido a la inclusión de proteínas y hacen posible la acción de compuestos y de fosfolipasas (Evers, 2004; Michalski & Januel, 2006).
Además el hecho de que las muestras procedentes de leches obtenidas en Junio no mostraran disminuciones en el contenido de PE, indican que existe una relación entre los cambios observados y la composición de la leche así como que PI y PS son componentes altamente sensibles a la alteración.

Este es el primer estudio centrado en el efecto de las altas presiones hasta 900 MPa en la fracción lipídica de la leche. Estudios posteriores deberán centrarse en determinar el agente causante de estas alteraciones, así como los mecanismos que se desencadenan por este tratamiento tan intenso.

4.4 Determinación de la posible alteración de la fracción lipídica de fórmulas infantiles en polvo y derivados lácteos con elevado contenido en CLA, durante su periodo de conservación.

4.4.1 Alteraciones en la fracción lipídica durante la conservación de fórmulas infantiles en polvo.

Los resultados aquí discutidos en la sección siguiente corresponden con el capítulo: "Changes in the Lipid Composition of Powdered Infant Formulas during Long-Term Storage".

Los análisis estadísticos aplicados a la composición en ácidos grasos de los dos tipos de fórmulas infantiles (término y crecimiento) durante un periodo de conservación de cuatro años empleadas en el estudio, no revelaron diferencias significativas entre ellos, lo que puede atribuirse a que ambos preparados, según la información proporcionada por el fabricante, fueron elaborados con la misma mezcla de aceites vegetales y con los mismos procedimientos tecnológicos. Estos resultados sugieren que las diferencias que pudieran existir

en cuanto a otros componentes entre ambas formulas infantiles no afectan a la composición lipídica durante la conservación.

Sin embargo, durante el almacenamiento se produjeron pérdidas significativas en la concentración en oleico durante el primer año de conservación, mientras que el contenido en linoleico se redujo únicamente durante el cuarto año de conservación, mientras que el linolénico lo fue en el primer año. Chavez-Servin *et al.* (2008) estudiando la evolución de la fracción lipídica durante la conservación de fórmulas infantiles comerciales en polvo, simulando una situación de empleo doméstico, observaron perdidas en los contenidos de linoleico y linolénico pero no en otros con mayor grado de insaturación como araquidónico o DHA. También se detectaron compuestos volátiles relacionados con la degradación oxidativa de los ácidos grasos (hexanal y heptanal) que fueron detectados en algunas de las muestras estudiadas. En cuanto al CLA este permaneció sin cambios significativos durante el periodo estudiado. El hecho de que únicamente se detecten variaciones en las concentraciones de oleico tras el primer año de conservación y de linoleico y linolénico al final del periodo de estudio, mientras que otros ácidos grasos de cadena corta y media no mostraron alteraciones, hace pensar que en este caso los efectos observados se deben a reacciones de carácter oxidativo y no a la acción de lipasas termoresistentes como se ha observado en otras partes del presente estudio. Trabajos previos llevados a cabo en leches infantiles enriquecidas en ácidos grasos poliinsaturados de cadena larga (LC-PUFA) durante un periodo de conservación de 15 meses a temperaturas de 25 y 37ºC (Romeu-Nadal *et al.*, 2007) y en leches UHT (control y de alto contenido en ácidos grasos insaturados) conservadas a 20ºC, con exposición a la luz y otras embotelladas con diferentes materiales que evitaran la permeabilidad al oxígeno y la exposición a la luz, durante 85 días, concluyen que los factores que favorecen el desarrollo de reacciones de oxidación como exposición a la luz, presencia de O_2, metales y la temperatura de almacenamiento, son factores determinantes en el desarrollo de las reacciones que contribuyen a la alteración de esta fracción.

En el presente estudio, las fórmulas infantiles estudiadas presentaron relativa estabilidad en su fracción lipídica hasta el último año de conservación, donde

tuvieron lugar las principales alteraciones de carácter oxidativo, reflejándose en disminuciones significativas en las concentraciones de ácido oleico, y pérdidas en el contenido de ácidos linoleico y linolénico y de tocoferoles.

4.4.2. Alteraciones durante la conservación de derivados lácteos de alto contenido en CLA.

Los resultados aquí disctutidos en la sección siguiente corresponden con el capítulo: "Fatty Acid and Conjugated Linoleic Acid (CLA) Isomer Composition of Commercial CLA-Fortified Dairy Products: Evaluation After Processing and Storage".

Las muestras analizadas de productos comerciales: Leche, leche en polvo (no comercial), leche fermentada, yogur, queso fresco y una bebida de mezcla de zumo y leche, elaborados y suministrados por el fabricante, a partir de leche desnatada a la cual se le adiciona un aceite de alto contenido en CLA (Tonalin). En leche, la composición obtenida mediante análisis de GC revela la presencia de un contenido significativo de grasa láctea, que varió según el lote, por lo que no fué posible hacer una comparación de los derivados lácteos mediante esta técnica. Aunque los lácteos y derivados son las principales fuentes naturales de CLA, sus concentraciones son relativamente bajas (0.1-1 g/ 100 g grasa) (Jensen, 2002) por lo que puede asumirse que la práctica totalidad es eliminado durante el desnatado de la leche y no influye en la distribución de isómeros de CLA en los derivados lácteos estudiados.

Durante el estudio se observaron algunos efectos debidos al procesado y que aún no siendo parte de la temática a desarrollar en esta parte del presente trabajo, presentan una relevancia altamente significativa en la temática y objetivos de la presente tesis, por lo que se discutirán brevemente en este apartado. Así, el análisis mediante Ag^{+}-HPLC reveló que se producían disminuciones significativas en isómeros minoritarios (C18:2 c11, t13 y C18:2 t8, c10) en leche en polvo, leche fermentada, yogures y la mezcla de zumo y

leche. También se observó un incremento significativo en el contenido de los isómeros *trans, trans* en leche en polvo.

Es conocido que los dobles enlaces *trans* son más estables que los *cis* y que el incremento en la concentración de compuestos *trans* por isomerización esta favorecido termodinámicamente por la temperatura y el tiempo de reacción (Bruice & Lightstone, 1999). Esto se confirma en trabajos previos en mantequillas sometidas a calentamiento de hasta 350ºC donde se produjo un aumento en los contenidos en ácido elaídico (C18:1 t9) por isomerización del oleico (Precht *et al.*, 1999; Herzallah *et al.*, 2005). En un estudio del efecto de los procesos de calentamiento y refinado de aceites de girasol se observó un aumento de los compuestos C18:2 *trans, trans* asociados con la temperatura de tratamiento (Juaneda *et al.*, 2003). Destaillats *et al.*(2005) encontraron que el tratamiento térmico severo de mantequillas (200ºC, 6h) provocaba isomerización de ruménico para dar C18:2 t8, c10 y aumento en el total de compuestos CLA *trans, trans*.

En cuanto al estudio de los posibles efecto durante el periodo de conservación, en los derivados lácteos comerciales enriquecidos en CLA, la cromatografía de gases mostró que al final del periodo de conservación de 5 semanas se produjo una tendencia hacia la disminución no significativa del contenido en los isómeros de CLA, RA y C18:2 t10, c12 en leche fermentada y en queso fresco. Los análisis llevados a cabo mediante Ag^{+}-HPLC mostraron una disminución significativa en la concentración del isómero C18:2 c11,t13 así como la ausencia de los compuestos C18:2 c9,c11 y C18:2 c10,c12 presentes en las muestras control. Este mismo comportamiento de los compuestos *cis, cis* se observó en derivado lácteo de mezcla de zumo y leche. Estudios previos llevados a cabo en leche enriquecida con aceites de alto contenido en CLA y posteriormente pasteurizadas y conservadas en refrigeración tres semanas, detectaron que el contenido en RA y otros isómeros minoritarios disminuyeron significativamente durante el periodo estudiado, concluyendo que la diminución en el contenido de estos compuestos pudiera ser atribuida a la acción microbiológica (Campbell *et al.*, 2003). Estos resultados coinciden con los reportados por Herzallah et *al*. (2005) que en leches pasteurizadas y en yogures conservados en refrigeración donde observaron una disminución

significativa en el contenido total de CLA que se atribuyó al desarrollo de reacciones oxidativas. Sin embargo, otros trabajos con leche de vaca rica en CLA, TVA y omega 3, por suplementación de la dieta con aceites de pescado y semillas extrusionadas de soja, no encontraron variaciones en el contenido de dichos ácidos grasos tras un periodo de conservación de 30 días en refrigeración (Dave *et al.*, 2002). Mailla et al. (2008) estudiando el posible efecto de la conservación (8 dias, 6ºC) en mantequillas con un perfil lipídico rico en ácidos grasos insaturados y CLA, observaron perdidas en elaídico, mientras que el CLA permaneció estable.

Por ello, es posible indicar que aunque comparativamente, los efectos del procesado industrial sobre la fracción lipídica de productos y derivados lácteos, son de mayor intensidad, la conservación en refrigeración en muestras donde la grasa ha sido sustituida por un aceite rico en CLA, las modificaciones encontradas van en la misma línea, con incrementos significativos de los isómeros minoritarios *trans, trans* a partir de los isómeros de conformación *cis,trans-trans,cis y cis,cis.*

CONCLUSIONES

5. CONCLUSIONES.

Relativas a:

1.- Desarrollo de métodos de análisis rápido de ácidos grasos mediante cromatografía de gases y de análisis de clases lipídicas mediante HPLC-ELSD.

1A. Se ha optimizado un procedimiento rápido por CG que puede emplearse como método rutinario en el análisis de ácidos grasos de distintas grasas y aceites alimentarios (vegetales, pescado, etc.) y especialmente para grasa de leche y derivados lácteos ya que en aproximadamente 17 minutos permite la suficiente resolución para la detección e identificación de ácidos grasos *trans*, isómeros de CLA y CLnA así como ácidos grasos omega 3.

1B. Se ha desarrollado una metodología por HPLC-ELSD para el análisis de clases lipidicas de grasa láctea que permite la detección y cuantificación de lípidos apolares y polares mediante inyección directa sin necesidad de técnicas previas de aislamiento y/o concentración. Este procedimiento es de especial interés para el análisis cuantitativo y cualitativo de fosfolípidos, pudiéndose distinguir dentro de este grupo, fosfatiletanolamina, fosfatidilinositol, fosfatidilserina, fosfatidilcolina y esfingomielinas, así como algunos compuestos de hidrólisis como el ácido fosfatidico y la lisofosfatidilcolina.

2.- Estudio de la mejora del perfil lipídico de leche mediante la suplementación con PUFAs de la dieta rumiantes, o mediante el empleo de bacterias lácticas con capacidad para producir CLA.

2A. El suplemento en la alimentación de vacas con semillas de lino y maíz, permite la mejora del perfil lipídico de la grasa de leche al disminuir el contenido en ácidos grasos saturados, principalmente palmítico, y un aumento en el contenido de los insaturados oleico y vacénico, y poliinsaturados linolenico e isómeros de CLA (ruménico y *trans,trans)*, aunque también conduce a un aumento en el contenido del isómero C18:1 t10. Igualmente la suplementación de la dieta de cabras con semillas de lino, conduce a un perfil en ácidos grasos de la leche más saludable, al disminuir los contenidos en palmítico, mirístico y laurico y aumentar el contenido oleico, ruménico, C18.2 t11,t13, esteárico y vacénico, aunque sin afectar en este caso el contenido en C18:1 t10.

2B. Ha sido posible la identificación de cinco cepas de los géneros *Bifidobacterium* y *Lactobacillus* con capacidad de producir CLA, principalmente ruménico, C18:2 t10, c12, C18:2 t9, t11 y C18:2 t10, t12, en leche desnatada con ácido linoleico como substrato, aunque para que puedan constituirse como una alternativa en la elaboración de derivados lácteos de alto contenido en CLA, es necesario llevar a cabo investigaciones que mejoren el rendimiento de su producción.

3.- Estudio del efecto del procesado sobre los ácidos grasos y fosfolípidos de la leche.

3A. Los tratamientos térmicos de esterilización en leche de vaca obtenida mediante suplementación de la dieta con PUFAs, provocan la isomerización del ácido linoleico en el isómero de CLA C18:2 t9, t11 y en el caso de tratamientos de alta pasterización (HTST) y microondas, reacciones de reordenamiento sigmatrópico dando lugar a un incremento en la concentración de ruménico. En la elaboración de derivados lácteos en polvo de alto contenido en CLA, el tratamiento por atomización conduce al incremento de los isómeros *trans*, *trans*.

3B. La homogenización a altas presiones de leche cruda en el rango de 50-350 MPa, no produce alteraciones en el contenido y perfil de ácidos grasos. Igualmente el empleo de altas presiones hidrostáticas hasta 900 MPa, no producen variaciones de esta fracción. Sin embargo, tratamientos en el rango de presiones de 250-900 MPa, provocan disminuciones en el contenido de fosfatidiletanolamina (PE), fosfatidilserina (PS) y fosfatidilinositol (PI).

4. Evolución de los fenómenos de alteración de los ácidos grasos durante el periodo de conservación en fórmulas infantiles en polvo, y derivados lácteos de alto contenido en CLA.

4A. Durante el periodo de conservación de fórmulas infantiles, el perfil de ácidos grasos permanece estable durante el primer año, si bien, superado ese

tiempo, tienen lugar alteraciones de carácter oxidativo que disminuyen las concentraciones de ácido oleico, y pérdidas en el contenido de ácidos linoleico y linolénico y de tocoferoles principalmente durante el ultimo año de conservación.

4B. Durante el proceso de conservación en refrigeración de derivados lácteos donde la grasa ha sido sustituida por un aceite rico en CLA, no se producen modificaciones significativas en la concentración de los isómeros mayoritarios C18:2 cis 9 trans 11 (RA) y C18:2 trans 10 cis 12, aunque se incrementan los isómeros minoritarios trans, trans a partir de los isómeros de conformación cis,trans-trans,cis y cis,cis.

5. Conclusión general

La suplementación de la dieta de rumiantes con semillas de lino y el empleo de bacterias lácticas en productos fermentados son estrategias válidas para el aumento del contenido en lípidos bioactivos, como PUFAs, los isómeros de CLA y ácido vacénico, en productos lácteos, en detrimento del contenido en SFA. Los tratamientos térmicos de esterilización, alta pasterización y microondas de la leche con perfil lipídico mejorado de forma natural, afectan al contenido y distribución de los PUFAs, especialmente a los isómeros de CLA, mientras que los tratamientos de altas presiones dismuyen al contenido en fosfatidiletanolamina, fosfatidilserina y fosfatidilinositol. En derivados y fórmulas lácteas, de prolongado período de conservación hasta su fecha de caducidad, en general se produce la disminución de ácidos grasos mono y poliinsaturados

en las etapas finales de estos períodos, sin descartar por otra parte el posible incremento de TFA.

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